REMARKS

Amendment to the Specification

The specification has been amended in accordance with Examiner's request and suggestions.

Nonstatutory double patenting rejection

Examiner has rejected claims 1-7 based on obviousness-type double patenting as being unpatentable over claims 1-7 of US Patent No. 6,649,165. The Applicant will timely file a terminal disclaimer in compliance with 37 CFR 1.321(c) to overcome this rejection.

Applicant's Declaration and other References Appended

Applicant's **Declaration** in support of this Response is attached hereto, with appendices, and is hereby incorporated by reference in its entirety as part of this Response. **Appendix A** to the Declaration is a research "Report" from Applicant's company MelTec. Where additional references cited in the Declaration are to short articles or to text found in Abstracts of such additional references, then those articles and Abstracts are appended in printed form as **Appendix B** to the Declaration. Other citations are to information directly accessible by the Examiner on-line and URL addresses for each of these citations can be found in the additional page appended to the Declaration entitled "**Appendix C** -- Declaration References with URL Addresses."

Other references cited in this Response are listed in the appended page entitled "**Response Appendix 1**. Response References," again with URL addresses included to provide the Examiner access to online materials. For instance, the reference Clark et al., 2005, is a 350+ page document, which Applicant has considered would be less burdensome on Examiner if presented through online access. Where the Response cites references that are short articles or Abstracts and these are part of Declaration

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Appendix B, then a notation to this effect in the listing of Response References will so direct the Examiner.

Section 112 claim rejections

The Examiner has rejected claims 1-7 under 35 U.S.C. §112, first paragraph, as being non-enabling for "the full breadth of soluble Fcγ receptors and a method of treating a patient with ALS." Further, Examiner states, "The specification does not enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the claimed invention commensurate in scope with these claims." (O.A., Par. 7). Applicant respectfully traverses Examiner's argument. First, the Examiner has not stated a proper basis for a non-enablement rejection. Second, the application is enabling and the Examiner has failed to show that it is not. Third, the invention upon filing was reasonably predictable to be operative. And fourth, such operativeness continues to be reasonably predictable in view of appropriate and relevant post-filing research and modern references.

1. The Examiner has not stated a proper basis for a non-enablement rejection.

The Examiner has failed to present a *bona fide prima facie* case that Applicant's disclosure is not enabling for soluble Fcγ receptors other than soluble FcγRIII. The Examiner has rejected claims 1-7 based on non-enablement under Section 112, arguing in general terms that the specification does not enable any person skilled in the art to make and use the claimed invention. The Examiner fails to show any specific steps in the manner and process of making and using the invention that are not taught, or are missing.

The Examiner blurs the issues of enablement and operativeness in arguing that the claimed method of treating a patient with ALS, more particularly by injection of soluble Fcγ-RI and Fcγ-RII receptors, is not supported. In one aspect, the Examiner is trying to rely on Section 112

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for an inoperativeness rejection; but, Examiner, needing more than doubt alone, must use references. However, the Examiner's references are not relevant evidence, because they do not discuss use of soluble Fcγ receptors (whereas numerous new references do in fact support the reasonableness of a therapeutic scheme according to the invention). In other aspects, the Examiner ignores clear statements in the specification in order to allege lack of guidance.

2. Overcoming Examiner's arguments of non-enablement.

The Applicant respectfully disagrees with the Examiner's assessment that the specification is non-enabling for a method of treating ALS by administering an effective amount of soluble Fcγ receptors, wherein said soluble Fcγ receptors bind to immunoglobulin in said patient to treat amytrophic lateral sclerosis, for at least the following reasons. To the contrary, the specification as filed enables a person skilled in the art to make and use the invention; specific guidance is given, and all necessary steps in the manner and process of making and using are taught. It is clear that the inventor had the invention fully in hand at the time of filing.

The Examiner admits that "the specification is enabling for a method of blocking the cytotoxic activity of FcγRIII-positive, ALS-specific cells in a patient with ALS using soluble FcγRIII." Further, Examiner admits that the specification discloses "that the blood of ALS patients contains mononuclear cells bearing CD16 and a different number of various other receptor proteins as listed in Table 1, and that the soluble Fcγ receptors block the cytotoxic activity of ALS-specific cells." (O.A., Par. 8).

Contrary to the Examiner's statement in Par. 9 of the Office Action, the specification indeed does provide sufficient guidance and direction with respect to soluble Fcγ receptors as broadly recited. The method of the invention is equally enabled in the specification for all classes (i.e., RI, RII and RIII) of soluble Fcγ receptors as much as for

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any one class of soluble FcγRIII receptors. The entire group of Fcγ receptors (all three classes) is identified in the disclosure, as well as the source and derivation of each class sufficient for a skilled molecular biologist to obtain any of the group (Specification, page 3, final paragraph et seq.), and an appropriate dosage range is given for an initial therapeutic scheme from which a skilled medical practitioner can derive a therapeutic scheme for each and every soluble Fcγ receptor in the group, i.e., 10-1,000 mg/kg for soluble Fcγ receptors. (see Specification, page 6). Further, methods of producing the full breadth of soluble Fcγ receptors have been known in the art since prior to the filing date sufficiently for a trained molecular biologist to obtain the required substances (e.g., see Sautes et al., 1994). And, Example 1 in Applicant's disclosure teaches broadly FcγRs produced from *E. Coli* fusion proteins.

The Examiner further states that the specification does not adequately teach how to effectively treat ALS or reach any therapeutic endpoint in patients by administering soluble Fcγ receptors, and that the Example 1 on page 8 of the specification "merely showed toleration test for soluble Fcγ preparations." (O.A., Par. 10). Applicant respectfully requests that the Examiner read Example 1 again and more closely. To the contrary, with regard to therapeutic regimen, Example 1 does not merely disclose a toleration test; rather, the Example provides guidance for a therapeutic scheme for the full breadth of soluble Fcγ receptors whereby one begins with a toleration test of 10-1,000 mg/kg, and then administers "subsequently 150 mg/weight kg daily, over a period of 5 days." (Example 1, page 8). Further, Applicant clearly discloses a range for adminstering the full breadth of therapeutic dosage of Fcγ receptors, including very specific guidance for adjusting dosage based on observed response,

> "Said substances will therefore be applied in the respective therapeutic dosage and after a toleration test in bolus [...]. Approximate values are the following concentrations,

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> for example: [...], for soluble Fcγ receptors (10 to 1,000 mg/kg), [...]
>
> However, an exact therapeutic dose can only be decided with respect to the individual case concerned. It depends, amongst other things, on the response of the Fcγ receptor-positive cells to the administration of the substances as indicated in claim 1 and on the individual toleration which may be determined by single bolus administrations (see example 1). Such response may e.g. be established by the determination of the number of such cellular forms in the blood or by in-vitro cytotoxicity assays." (Specification at page 6, last paragraph and page 7, first two paragraphs)

Contrary to the view of the Examiner, how to effectively treat ALS and reach a therapeutic endpoint is specifically addressed in the disclosure, as follows (emphasis supplied),

> "2. Based on the knowledge, according to the invention, of the ALS specific surface characteristics, i.e. the combinatorial receptor patterns of these cells (see Table 1), it is possible to ascertain before each therapy whether these cells are present at all, or, after their isolation and *in-vitro* testing, to determine whether they exhibit any cytotoxic, in particular, neurotoxic, activity.
>
> 3. Based on the knowledge, according to the invention, of the cell surface characteristics of these cells, the therapeutical success can not only be determined clinically, but also on a cellular level, with the therapy according to claim 1 in full progress, by the examination of blood samples for a therapy-related decrease in the number, or even a total elimination of the Fcγ receptor-positive cellular forms from the blood stream.
>
> 4. Based on the findings of above step 3, the dosage of the administered substances may be adapted accordingly, i.e. increased or decreased, depending on whether or not there is a response in the Fcγ receptor-positive cellular forms.
>
> 5. [...] a disease-specific cell parameter for ALS , it is possible to correlate the clinical course of the disease precisely with this parameter, so that [...] a re-increase of the [...] cells will allow exact determination of the time for a further therapy cycle." (see Specification at page 7, numbered paragraphs 2-5).

A therapeutic endpoint is specifically disclosed in quoted paragraph #3 above. This guidance on how to treat, together with the

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specified endpoint, provides adequate teaching for a skilled medical doctor to treat ALS patients with the method of the claimed invention.

Finally, the Examiner argues (O.A., par. 11) that the claimed invention cannot be practiced without undue experimentation; however, the Examiner fails to provide any specific examples of where any experimentation would be required. In molecular biology and medical practice, adjustments in values are normal and expected and within the ordinary skills of these professionals in each field to fit individual conditions; this is not undue and no "invention" is required. Example 1 and the "solution advantage" step #3 in Applicant's specification (page 7) provide adequate guidance and direction so that adjustments can proceed without undue experimentation. The patent laws do not require applicant to teach at the cookbook recipe level.

3. No legitimate question of operativeness exists.

Based on the subject matter, the *in vitro* studies cited, and the other research results disclosed in the specification, the operativeness of the invention is reasonably predictable as of the date of filing of the application

The invention relates to use of substances for the selective suppression, destruction or selective functional blocking of ALS-specific, Fcγ receptor-positive cellular forms, or blocking or functional inactivation of Fcγ receptors, by infusion or injection of soluble Fcγ receptors into the serum of ALS patients. The therapeutic strategy is to bind the gamma region of the IgG1 and IgG3 in the serum of patients with these introduced soluble Fcγ receptors, for the purpose of preventing the FcγR-positive macrophage cells from being activated by said IgG1 and IgG3.

That the ALS disease mechanism involves FcγRIII-positive macrophage activation is supported by the inventor's research results disclosed in the specification. These results show that ALS disease-specific, combinatorial patterns exist on the cell surfaces of ALS

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patients, and further that the FcγRIII sub-class of Fcγ receptors (the subclass known as CD16) is associated on these particular cell surfaces of ALS patients with the abnormal presence of CD2, CD56, HLA-DR, and/or CD7 and the abnormal absence of CD38 and/or CD62L (see Table 1 in Specification; also see Table 4 in Appendix A of Applicant's Declaration, attached hereto).

As noted above, the Examiner admits that the specification discloses "that the blood of ALS patients contains mononuclear cells bearing CD16 and a different number of various other receptor proteins as listed in Table 1, and that the soluble Fcγ receptors block the cytotoxic activity of ALS-specific cells." (O.A., Par. 8).

A therapeutic treatment strategy does not have to be proven to be a "cure," nor must it be proven to be optimally effective, to be deserving of patent protection, so long as the method can be reasonably predicted to have a therapeutic effect.

Operativeness of Applicant's invention, in terms of the efficacy of the claimed method of treating a patient with ALS, was reasonably predictable at time of filing, based on *in vitro* studies disclosed in the specification at pages 5-6. At the time of filing, it was reasonably predictable in light of the specification that amyotrophic lateral sclerosis (ALS) could be treated by administering soluble Fcγ-RI, Fcγ-RII, and/or Fcγ-RIII receptors to effect Fc receptor blockade. Reasonably logical and plausible mechanisms for soluble FcγR receptors to impact ALS disease through immunopathological mechanisms existed and were apparent to the Applicant.

The existence of specific cellular forms and immunnopathomechanism has been confirmed by examinations disclosed by the Applicant in the specification. The blood of ALS patients contain mononucleic cells bearing Fcγ receptors on their cellular surfaces, additionally or simultaneously bearing a different number of various other receptor proteins which exhibit an unusual form of cellular surface activation in combination with Fcγ receptors, such as, for example, with FcγRIII (CD16)

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(see specification, Table 1, and page 5, third full paragraph). These cells, after being isolated from the blood of ALS patients, were co-cultivated with nerve cells and serum of ALS patients to display a cytotoxic activity, which activity could be blocked by soluble Fcγ receptors at a concentration of between 10 and 60 μmol (see Applicant's Specification at page 6, second paragraph).

As detailed in the inventor's Declaration, the ALS-specific immune cells are characterized by specific protein combinations, which combinations identify Fcγ receptor as key proteins. The report attached to the Declaration (Appendix A) provides results of comparison between occurrence of Fcγ receptor in the immune cells of normal individuals versus occurrence in immune cells of patients with ALS. Applicant's technology of toponomic fingerprinting (TF) allows Applicant to identify a characteristic collection of combinatorial protein patterns (CPP) which contain protein clusters that are highly specific for a cell type, for a functional state of a cell (e.g., as in disease), or for a tissue. The present invention provides for a therapeutic method deriving directly from this important TF assessment and knowledge as it pertains to ALS-diseased cells, i.e., the value of interrupting the CD-16 receptor activation within these ALS-specific protein clusters by tying up IgG gamma-chain sites in serum by using any of the classes of soluble FcγRs in serum, creating the result that the serum-borne IgG is thereupon diminished in its ability to bind those cell-surface CD-16 receptors existing in the cell-surface protein network.

Table 4 of Applicant's Report attached to Applicant's Declaration (see page 17 of Declaration Appendix A), provides a list of ALS-specific motifs, i.e., most-significant abnormal, cell-surface, protein clusters in ALS. Table 5 (page 20 of Appendix A), provides a comparison of normal versus disease. CD16 (FCγRIII) was the leading protein found to be present with significant frequency on the surface of macrophage cells in ALS patients. While the data indicate that CD16 is the leading protein in both healthy and ALS blood cells, the motifs were found to be

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inherently different See Declaration, paragraph 13). ALS-specific cell surface protein clusters indicate presence of abnormal cell surface differentiation. Moreover, CD16 can be indicated as a valid peripheral biomarker for ALS and a basis for CD16-oriented modification therapy (see Declaration, paragraph 13).

A medical conclusion from this evidence, which was reached by the inventor at the time of filing and which would be reasonably apparent to others skilled in the relevant art when armed with the disclosure, is that these abnormally patterned, ALS-disease-specific macrophage cells are directly related to the disease mechanism, i.e., in ALS patients these abnormal, FcγRIII receptor-positive macrophages are being activated in a manner and rate that is slowly killing the patient. Further, it is logical and reasonable to predict, solely from the inventor's research results disclosed in the specification, that any substance introduced to the serum that would selectively bind the constant gamma chain of immunoglobulin in the serum, i.e., any soluble Fcγ receptor, would thereby selectively deprive the FcγRIII receptors on the ALS-disease-specific immune cells from binding with said immunoglobulin, and thus would block activation of these disease-specific immune cells with predictable benefit to the patient thereby (see Declaration, Par. 14).

The Examiner cites no specific reason why the full breadth of soluble Fcγ receptors being engaged in the blocking of disease-specific immune cells does not, would not or could not work, nor does Examiner specify evidence to justify Examiner's arbitrary drawing of a line between the efficacy of soluble FcγRIII and the efficacy of FcγRI and FcγRII for the same purposes and successful effect of the invention. Examiner cites Nakamura et al. for the position that each of the three classes of Fcγ receptors encodes by different genes and has different affinity for IgG; however, the Examiner makes no further specific connection between this assertion and its relevance to either enablement or operativeness. In fact, nothing in Nakamura et al.

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speaks against the operativeness of the invention. The structure of the full breadth of Fcγ receptors is similar with respect to the structure of the γ-chain constant fragment in the compounds of each class of receptor. The transmembrane form of FcγRIII associates with the γ-chain subunit of FcγRI, and it has structural similarity with FcγRI and FcγRII (http://www.biology-online.org/dictionary/fc_receptors). Thus, soluble FcγRI, FcγRII and FcγRIII present substantially the same capability with respect to the invention insofar as they all will bind the gamma(γ)-chain region on IgG in serum. Therefore, all three classes of these compounds operate in similar fashion to prevent blood-borne IgG from subsequently activating the FcγRIII-positive, ALS-disease-specific immune cells (see Applicant's Declaration at Par. 7, 14 and 15).

The Examiner states (O.A., Par. 10) that, "It is not clear that reliance on example 1 and the fact that ALS-specific cells having Fcγ receptors [?] accurately reflect the efficacy of the claimed method of treating a patient with ALS." Citing McGeer et al., the Examiner asserts that "the state of the art recognizes that the mechanisms and processes responsible for the selective loss of motor neurons are still unknown and there is no cure or effective treatment presently exist[ing]." Applicant respectfully submits that the McGeer article as cited by the Examiner is not a reasonable reference against operativeness in the present case. As pointed out in the inventor's attached Declaration, McGeer et al. reviews a set of therapeutic schemes that neither includes nor is related to the therapeutic strategy of the invention.

Similarly, the remaining two references cited by the Examiner, Dalakas et al. and Rudnicki et al., have no logical bearing on potential applicability of treatment of ALS by injecting soluble FcγR receptors. With regard to Rudnicki et al. (1987) it is not relevant that, many years prior to the Applicant's filing of Applicant's

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invention, these researchers reported, for one ALS patient with increased serum IgG, that lowering the serum concentration of IgG with immuno-supressive agents and plasmaphoresis was not effective in treating the patient. This has no logical bearing because (a) Rudnicki et al. only reduced postpheresis serum IgG to 876 mg/dl which is still within a normal range and, most importantly, (b) there is no indication that Rudnicki et al. reduced activation of the FcγRIII receptor-positive, ALS-disease-specific, immune cells. Similarly, the conclusion of Dalakas et al., i.e., that high-dose intravenous immunoglobulin is not an effective treatment for ALS, in no way contradicts Applicant's results or therapeutic strategy according to the invention. Indeed, if IgG binding to the FcγRIII-positive, ALS-disease-specific immune cells operates to activate these cells and to cause damage to the patient, then one would expect that increasing IgG levels in the blood, in the absence of selectively disabling the binding of the IgG to these particular Fcg receptors embedded on the surface of the immune cell, would worsen the patient's condition. In other words, the Dalakas et al. method of "blocking" Fcγ receptors with IgG-binding would not be predicted to be very effective if this binding step in fact operates to activate the ALS-disease-specific immune cells cells. Exactly to the contrary, Applicant's therapeutic strategy according to the invention is to prevent such IgG-binding at the embedded Fcγ receptor sites on the ALS-disease-specific immune cell surfaces by earlier binding this IgG in serum using the full breadth of soluble Fcγ receptors, in order to specifically prevent activation of these disease-specific cells.

In assessing operativeness of Applicant's invention, then, the Examiner has not only incorrectly assessed the state of the art in ALS research with regard to soluble Fcγ receptors, the Examiner has applied references that are not logically relevant to the question of predicting operativeness of Applicant's invention.

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4. Biomedical research post-filing continues to support predictability of operativeness.

Continuing medical research into one or more immunopathological mechanisms supports the predictability of operativeness of Applicant's invention, such as, for instance, blocking of the Fcγ receptor-mediated cytotoxic activity of the ALS-specific Fcγ receptor-positive immune cells, so as to prevent the direct or indirect pathological effects of these mechanisms on the motoneuron system in ALS patients.

It is recognized that Fcγ receptors play critical roles in autoimmune diseases (Alerio, 2006), for instance through phagocytosis and subsequent oxidative stress. Evidence of increased oxidative stress damaging tissues and DNA of ALS patients has been reported (Ferrante et al., 1997). Over the past 40 years, oxidative stress has been one of the six major etiological assumptions underlying ALS clinical trials (Clark et al., 2005, p. 147). Aggregation of receptors (FcγRs) for IgG leads to a number of cellular responses, including endocytosis and phagocytosis, which in turn stimulate cytokine release and activating NADPH oxidative bursts (Alerio, 2006). Also, FcγRs are now thought to participate in IgG uptake into motor neurons as well as IgG-mediated increases in intracellular calcium and acetylcholine release from motor axon terminals (Mohamed, 2002), and details are emerging on how increasing intracellular calcium is associated with cell death (Brevnova et al., 2004).

Recent studies using FcR-deficient mice reveal that the development of autoimmune diseases depends upon FcγRs and that FcγRs mediate and control the effector functions of IgG antibodies and the autoimmunity-tolerance balance in the periphery (see Applicant's Declaration, citing Suates-Fridan, 2003). FcγRI uses the γ-chain to recruit tyrosine kinases to initiate signal transduction. The γ-chain is a small membrane-anchored protein that contains an immunoreceptor tyrosine activation motif (ITAM). It assists signal transduction for a number of Fc receptors receptor including FcγRI, FceRI, FcaRI and FcγRIII

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(Alirio, 2006). Immune cells (e.g., macrophages) with impaired FcγR function show impaired phagocytosis, owing to co-engagement of FcγRs during phagocytosis effectively triggering activation of the Src tyrosine-kinase family, which phosphorylates ITAM (Nakamura et al., 2005, p. 175).

Clinical trials have not yet tested administration of soluble FcγRs for ALS. To date, immunosuppressant strategies in clinical trials have been only cyclosporine, prednisolone, azathiprene, cylophosphadine and radiation (Clark et al., 2005). In 2006, soluble Fcγ receptors are still categorized as "new leads" by the ALS Therapy Development Foundation [see attached ALS-TDF Web page].

Therefore, the growing body of biomedical research provides evidence and support for immunopathological mechanisms implicated in ALS disease that call for implementing Applicant's invention in clinical trials for ALS patients, therapies that would include using the full breadth of soluble Fcγ receptors to block activation of FcγRIII-positive ALS disease-specific immune cells; thus, showing the continuing, reasonable predictability of the operativeness of Applicant's invention.

For the foregoing reasons, Applicant respectfully traverses Examiner's rejection of Claims 1-7. Applicant respectfully requests reconsideration and withdrawal of the foregoing rejection.

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CONCLUSION

In view of the foregoing Remarks and attached Declaration of the inventor and other supporting references, Applicant believes that the present application is in condition for allowance.

The Examiner is encouraged to telephone the undersigned attorney to discuss any matter that would expedite allowance of the present application.

Respectfully submitted,

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ATTACHMENTS: RESPONSE APPENDIX 1 -- RESPONSE REFERENCES
DECLARATION OF INVENTOR
DECLARATION APPENDIX A
DECLARATION APPENDIX B
DECLARATION APPENDIX C

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Filed: September 12, 2003
Group Art Unit: 1644

RESPONSE APPENDIX 1 -- RESPONSE REFERENCES

Sautes et al., 1994. [see Declaration Appendix B for Abstract]
Recombinant soluble Fc gamma receptors: production, purification and biological activities. Sautes C, Galinha A, Bouchard C, Mazieres N, Spagnoli R, Fridman WH. J Chromatogr B Biomed Appl. 1994 Dec 9;662(2):197-207.
http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&list_uids=7719476&dopt=Abstract

Biology-online, 2006: [see copy of web page attached]
http://www.biology-online.org/dictionary/fc_receptors

Alirio, 2006: [see copy of web page attached]
http://www.med.nus.edu.sg/phys/Projects_FcgR_Alirio.htm

Ferrante et al., 1997: [copy of Abstract attached]
Evidence of increased oxidative damage in both sporadic and familial amyotrophic lateral sclerosis. Ferrante RJ, Browne SE, Shinobu LA, Bowling AC, Baik MJ, MacGarvey U, Kowall NW, Brown RH Jr, Beal MF. *J Neurochem.* 1997 Nov;69(5):2064-74.
http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=retrieve&db=pubmed&list_uids=9349552&dopt=Abstract

Clark et al., 2005: [359 page document; accessible online]
"Amyotrophic Lateral Sclerosis: A report on the state of research into the cause, cure, and prevention of ALS," June 2005; J. Clark, C. Pritchard, S. Sunak; Prepared for the Dept. of Public Health, State of Massachusetts by the ALS Therapy Development Foundation;
http://www.als.net/docs/ALSReport.pdf

Mohamed et al., 2002: [copy of Abstract attached]
Immunoglobulin Fc gamma receptor promotes immunoglobulin uptake, immunoglobulin-mediated calcium increase, and neurotransmitter release in motor neurons. Mohamed HA, Mosier DR, Zou LL, Siklos L, Alexianu ME, Engelhardt JI, Beers DR, Le WD, Appel SH. *J Neurosci Res.* 2002 Jul 1;69(1):110-6.
http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&list_uids=12111822&dopt=Citation

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Filed: September 12, 2003
Group Art Unit: 1644

(RESPONSE APPENDIX 1, cont. -- RESPONSE REFERENCES)

Brevnova et al., 2004: [see article attached hereto]
Overexpression of human KCNA5 increases IK(V) and enhances apoptosis; Brevnova, E. E., O. Platoshyn, S. Zhang, and J. X.-J. Yuan. Am J Physiol Cell Physiol 287: C715-C722, 2004.
http://ajpcell.physiology.org/cgi/content/full/287/3/C715

Sautes-Fridman et al., 2003: [see Declaration Appendix B for article]
Fc Gamma Receptors: A magic link with the outside world. Catherine Sautes-Fridman, Lydie Cassard, Joel Cohen-Solal and Wolf-Herman Fridman. *ASHI Quarterly, 4th Quarter*, 2003, p. 148-151
http://www.ashi-hla.org/publicationfiles/ASHI_Quarterly/27_4_2003/Fc_Gamma_Rec.pdf

Nakamura et al., 2005. [Examiner cited]
Fc receptor targeting in the treatment of allergy, autoimmune diseases and cancer. Nakamura, A., K. Akiyama, and T. Takai. Expert Opin. Ther. Targets (2005) 9(1):169-190
http://www.ashley-pub.com/doi/abs/10.1517/14728222.9.1.169;jsessionid=ihuMe4IGGJW9NTjBlL?cookieSet=1&journalCode=ett

ALS Web page: [see Declaration Appendix B; one page]
http://www.als.net/research/treatments/treatmentDetail.asp?treatmentID=993

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Fc receptors

(Science: immunology) Receptors for the Fc portion of immunoglobulins.

Fc_R (30 kD) is the receptor for serum and secretory IgA1 or IgA2 and is expressed on most myeloid cells and subpopulations of T and B-cells. Fc_RI (CD64) High affinity receptor (72 kD on gels) for monomeric IgG1 found on monocytes, macrophages and some neutrophils. The extracellular portion has three immunoglobulin superfamily C2 domains, in contrast to Fc_RII, Fc_RIII that have only two. Involved in antibody dependent cell killing and in clearance of immune complexes. Fc_RII (CD32) Low affinity receptor 40 kD) for aggregated IgG that exists in several sub types coded by three closely related genes, A, B and C. All forms are found on monocytes, the B forms (that are alternatively spliced) are found on B-cells, the A and C forms are present on neutrophils. Binding of aggregated IgG will trigger phagocytosis and the oxidative burst in neutrophils. Fc_RIII (CD16) is the low affinity receptor (50-80 kD on gels) for aggregated IgG. It is found in transmembrane and GPI linked forms. The transmembrane form associates with the _ subunit of Fc_RI or the TCR_ chain and on B-cells with the _ chain of Fc_RI. Has structural similarity with Fc_RI, Fc_RII and Fc_RI_. Binding of aggregated IgG or IgG antigen complexes mediates phagocytosis or antibody dependent cellular cytotoxicity. Fc_RI is a heteromeric high affinity receptor for IgE found on mast cells and basophils. The _ chain (45-65 kD on gels, 25 kD of polypetide) is N glycosylated and has two immunoglobulin C2 loops in addition to the transmembrane domain, the _ chain (32 kD) has four transmembrane domains, the _ subunit is a homodimer (8 kD monomer) identical to the _ subunit of CD16 and has similarity with _ and _ chains associated with the T-cell receptor. Binding of antigen to the IgE Fc_R complex triggers the release of histamine and various inflammatory mediators. Fc_RIIa & b CD23) Low affinity receptor (45 kD) for IgE. Both a and b are present on mature B-cells, the b form on monocytes, IL-4 activated macrophages, eosinophils, platelets and dendritic cells. The protein has a C type lectin domain that mediates IgE binding and can be cleaved from the membrane to yield an active soluble form.

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ALIRIO, 2006

Molecular mechanisms used by Fc-gamma receptors to trigger physiological responses.

Receptors for the constant region (Fc) of immunoglobulins play a pivotal role linking the humoral and cellular arms of the immune system. On leukocytes, aggregation of receptors (FcgRs) for immunoglobulin G (IgG) leads to a number of cellular responses including the internalisation of immune complexes (endocytosis), and/or of opsonised large particles/cells (phagocytosis). IgG -triggered Endocytosis or phagocytosis, stimulate a number of down-stream events including cytokine and matrix-protease release, activation of the NADPH oxidative burst, the production of eicosanoids (leukotriens and prostaglandins) and cellular migration. It also results in antigen presentation in association with either MHC Class I or II, resulting in CD8+ or CD4+ T cell activation respectively. In contrast, internalisation of apoptotic cells results in either no antigen presentation (degradation) or to presentation in the absence of activating co-receptors which induces T cell anergy and self tolerance. Fc g receptors specific for IgG (FcgRs) have been implicated in the pathogenesis of several diseases, in particular in inflammatory autoimmune diseases such as, systemic lupus erythromatous and rheumatoid arthritis. These Fc receptors, therefore, play critical roles in host defense mechanisms against invading pathogens, in auto-immune diseases and in cancer surveillance.

To mediate cell activation, the Fc-receptor initiates intracellular signalling cascades through the recruitment and activation of non-receptor tyrosine kinases. The cytoplasmic tail of the high affinity receptor for IgG, FcgRI, contains no recognised motif capable of activating tyrosine kinases. This receptor must recruit an accessory molecule to initiate signal transduction through the recruitment of non-receptor tyrosine kinases and the g chain has been shown to fulfil this role. The g chain exists as a homodimer being a small 7kDa membrane anchored protein that contains an immunoreceptor tyrosine activation motif (ITAM). It acts as the accessory molecule for signal transduction for a number of receptor including FcgRI, FceRI, FcaRI and FcgRIII. We have recently reported that, in interferon-g (IFNg) primed U937 cells, the g chain functionally couples FcgRI to a novel signalling pathway that involves the sequential activation of PI3-kinases, phosphatidyl choline phospholipase D (PLD) and sphingosine kinase.This pathway is necessary for efficient intracellular trafficking of FcgRI-internalised immune complexes to lysosomes for degradation the release of calcium from intracellular stores and the activation of the NADPH oxidase.

Our aim is to further investigate the molecular mechanisms that control FcgR-triggered physiological responses, including the release of enzymes from internal stores (degranulation), matrix metalloproteinase (MMP) release, cytokine release, the generation of leukotriens and prostaglandins (eicosanoids), internalisation, and antigen presentation, in inflammatory models, in order to identify key signalling molecules as target for novel therapeutic to treat inflammatory and autoimmune conditions.

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Evidence of increased oxidative damage in both sporadic and familial amyotrophic lateral sclerosis.

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Some cases of autosomal dominant familial amyotrophic lateral sclerosis (FALS) are associated with mutations in the gene encoding Cu/Zn superoxide dismutase (SOD1), suggesting that oxidative damage may play a role in ALS pathogenesis. To further investigate the biochemical features of FALS and sporadic ALS (SALS), we examined markers of oxidative damage to protein, lipids, and DNA in motor cortex (Brodmann area 4), parietal cortex (Brodmann area 40), and cerebellum from control subjects, FALS patients with and without known SOD mutations, SALS patients, and disease controls (Pick's disease, progressive supranuclear palsy, diffuse Lewy body disease). Protein carbonyl and nuclear DNA 8-hydroxy-2'-deoxyguanosine (OH8dG) levels were increased in SALS motor cortex but not in FALS patients. Malondialdehyde levels showed no significant changes. Immunohistochemical studies showed increased neuronal staining for hemeoxygenase-1, malondialdehyde-modified protein, and OH8dG in both SALS and FALS spinal cord. These studies therefore provide further evidence that oxidative damage may play a role in the pathogenesis of neuronal degeneration in both SALS and FALS.

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Immunoglobulin Fc gamma receptor promotes immunoglobulin uptake, immunoglobulin-mediated calcium increase, and neurotransmitter release in motor neurons.

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Receptors for the Fc portion of immunoglobulin G (IgG; FcgammaRs) facilitate IgG uptake by effector cells as well as cellular responses initiated by IgG binding. In earlier studies, we demonstrated that amyotrophic lateral sclerosis (ALS) patient IgG can be taken up by motor neuron terminals and transported retrogradely to the cell body and can alter the function of neuromuscular synapses, such as increasing intracellular calcium and spontaneous transmitter release from motor axon terminals after passive transfer. In the present study, we examined whether FcgammaR-mediated processes can contribute to these effects of ALS patient immunoglobulins. F (ab')(2) fragments (which lack the Fc portion) of ALS patient IgG were not taken up by motor axon terminals and were not retrogradely transported. Furthermore, in a genetically modified mouse lacking the gamma subunit of the FcR, the uptake of whole ALS IgG and its ability to enhance intracellular calcium and acetylcholine release were markedly attenuated. These data suggest that FcgammaRs appear to participate in IgG uptake into motor neurons as well as IgG-mediated increases in intracellular calcium and acetylcholine release from motor axon terminals.

MeSH Terms:

- Acetylcholine/biosynthesis
- Acetylcholine/secretion
- Amyotrophic Lateral Sclerosis/metabolism
- Animals
- Calcium/metabolism*

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Overexpression of human *KCNA5* increases $I_{K(V)}$ and enhances apoptosis

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Brevnova, Elena E., Oleksandr Platoshyn, Shen Zhang, and Jason X.-J. Yuan. Overexpression of human *KCNA5* increases $I_{K(V)}$ and enhances apoptosis. *Am J Physiol Cell Physiol* 287: C715–C722, 2004. First published May 12, 2004; 10.1152/ajpcell.00050.2004.— Apoptotic cell shrinkage, an early hallmark of apoptosis, is regulated by K^+ efflux and K^+ channel activity. Inhibited apoptosis and downregulated K^+ channels in pulmonary artery smooth muscle cells (PASMC) have been implicated in development of pulmonary vascular medial hypertrophy and pulmonary hypertension. The objective of this study was to test the hypothesis that overexpression of *KCNA5*, which encodes a delayed-rectifier voltage-gated K^+ (Kv) channel, increases K^+ currents and enhances apoptosis. Transient transfection of *KCNA5* caused 25- to 34-fold increase in KCNA5 channel protein level and 24- to 29-fold increase in Kv channel current ($I_{K(V)}$) at +60 mV in COS-7 and rat PASMC, respectively. In *KCNA5*-transfected COS-7 cells, staurosporine (ST)-mediated increases in caspase-3 activity and the percentage of cells undergoing apoptosis were both enhanced, whereas basal apoptosis (without ST stimulation) was unchanged compared with cells transfected with an empty vector. In rat PASMC, however, transfection of *KCNA5* alone caused marked increase in basal apoptosis, in addition to enhancing ST-mediated apoptosis. Furthermore, ST-induced apoptotic cell shrinkage was significantly accelerated in COS-7 cells and rat PASMC transfected with *KCNA5*, and blockade of KCNA5 channels with 4-aminopyridine (4-AP) reduced K^+ currents through KCNA5 channels and inhibited ST-induced apoptosis in *KCNA5*-transfected COS-7 cells. Overexpression of the human *KCNA5* gene increases K^+ currents (i.e., K^+ efflux or loss), accelerates apoptotic volume decrease (AVD), increases caspase-3 activity, and induces apoptosis. Induction of apoptosis in PASMC by *KCNA5* gene transfer may serve as an important strategy for preventing the progression of pulmonary vascular wall thickening and for treating patients with idiopathic pulmonary arterial hypertension (IPAH).

potassium ion channel; pulmonary hypertension

APOPTOSIS REGULATES CELL HOMEOSTASIS by removal of excess cells or cells with genetic damage and developmental mutations (49). Dysfunction or abnormal regulation of this process has been implicated in atherosclerosis, cancer, neurodegenerative disorders, and pulmonary vascular disease (12, 28, 29). At the cellular and molecular levels, apoptosis is characterized by a distinct series of morphological and biochemical changes that include cell shrinkage, caspase activation, and DNA fragmentation (13, 49).

Apoptotic cell shrinkage or volume decrease, an early hallmark of apoptosis, is a necessary prerequisite for the programmed cell death to occur (5, 11, 21, 24). Cell volume is primarily controlled by intracellular ion homeostasis; thus ion transport across the plasma membrane is important for the regulation of cell volume (20, 24). K^+ is the dominant cation in the cytoplasm (~140 mM) and thus plays a critical role in maintaining cell volume. Opening of sarcolemmal K^+ channels increases efflux or loss of cytoplasmic K^+ and induces apoptotic volume decrease (AVD), whereas closure or downregulation of K^+ channels decelerates apoptotic cell shrinkage and attenuates apoptosis (4, 5, 11, 18, 21, 23, 36, 38, 40, 41). In addition to its role in the control of cell volume, maintenance of a high cytosolic K^+ concentration ($[K^+]_c$) is required for suppression of caspases and nucleases (14), the final mediators of apoptosis (13, 49). Therefore, enhanced K^+ efflux is an essential mediator not only of early apoptotic cell shrinkage but also of downstream caspase activation and DNA fragmentation (24).

Pulmonary vasoconstriction and vascular remodeling are major causes for the elevated pulmonary vascular resistance in patients with idiopathic pulmonary arterial hypertension (IPAH). Pulmonary vascular remodeling is characterized by a combined adventitial, medial, and intimal hypertrophy. The pulmonary artery medial hypertrophy is mainly due to increased proliferation and/or decreased apoptosis of pulmonary artery smooth muscle cells (PASMC) (28, 29, 31, 35).

Downregulation and dysfunction of voltage-gated K^+ (Kv) channels in PASMC have been implicated in animals with hypoxia-mediated pulmonary hypertension (8, 15, 26, 33, 38, 44) and patients with IPAH (42, 46). The decreased Kv channel activity not only causes pulmonary vasoconstriction by inducing membrane depolarization and increases in cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$) in PASMC (43) but also contributes to pulmonary vascular medial hypertrophy by inhibiting apoptotic cell shrinkage and apoptosis (48).

KCNA5 (Kv1.5) is a pore-forming α-subunit that forms hetero- or homotetrameric Kv channels in many cell types including vascular smooth muscle cells (3, 8, 47). Normal expression and function of KCNA5 channels in PASMC are necessary for the regulation of resting membrane potential and pulmonary vascular tone (3, 43). It has been reported that KCNA5 channel expression is downregulated and Kv currents are inhibited in PASMC from animals and patients with hypoxia-mediated pulmonary hypertension (3, 27, 38) and IPAH (46). In vivo gene transfer of *KCNA5* with an adenoviral vector can inhibit hypoxia-mediated pulmonary arterial medial hypertrophy (27), suggesting that enhancing KCNA5 protein expression is a potential therapeutic approach for pulmonary arterial hypertension. This study was designed to test the hypothesis that overexpression of human *KCNA5* gene, in addition to causing pulmonary vasodilation due to increased Kv channel current ($I_{K(V)}$) and subsequent membrane hyperpolarization,

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enhances apoptosis in PASMC, which may contribute to the regression of PASMC hypertrophy and hyperplasia in pulmonary hypertension.

MATERIALS AND METHODS

Cell preparation and culture. All animal procedures in this study conform to the "Guiding Principles for Research Involving Animals and Human Beings" of the American Physiological Society. PASMC were prepared from pulmonary arteries of male Sprague-Dawley rats (43). Briefly, the isolated pulmonary arteries were incubated for 20 min in Hanks' balanced salt solution containing 1.5 mg/ml collagenase (Worthington Biochemical). Adventitia and endothelium were carefully removed after the incubation. The remaining smooth muscle was then digested with 1.5 mg/ml collagenase and 0.5 mg/ml elastase (Sigma) at 37°C. Approximately 45–50 min later, PASMC were sedimented by centrifugation, resuspended in fresh media, and placed onto petri dishes or coverslips. The monkey kidney COS-7 cells (American Type Culture Collection, Manassas, VA) and rat PASMC were both cultured in high-glucose (4.5 g/l) DMEM supplemented with 10% fetal calf serum (FCS), 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (BioFluids) and incubated in 5% CO_2 at 37°C in a humidified atmosphere.

Constructs. In the *KCNA5*-pBK construct (kindly provided by Dr. M. Tamkun from Colorado State University, Fort Collins, CO), the coding sequence of the human *KCNA5* gene was subcloned into *Xba*I and *Kpn*I sites of multiple cloning site (MCS) of the phagemid expression vector pBK-CMV (Stratugene). For electrophysiological experiments, a *KCNA5*-GFP construct was designed to visualize the transfected cells. In the *KCNA5*-GFP construct, the coding sequence of the human *KCNA5* gene was subcloned into *Eco*RI and *Xba*I sites of MCS of the pCMS-EGFP mammalian expression vector (Clontech). In the pCMS-EGFP vector, the EGFP gene [which encodes the enhanced green fluorescent protein (GFP), a red-shifted variant of wild-type GFP from *Aquorea victoria*] is expressed separately from the gene of interest and is used as a transfection marker.

Transfection of KCNA5. COS-7 cells and rat PASMC were transiently transfected with the expression constructs by using Lipofectamine reagent according to the manufacturer's instruction. Briefly, cells were first split and then cultured for 24 h. Transfection was performed on 40–80% confluent cells at 37°C in serum-free Opti-MEM I medium (Invitrogen) with 1.6 μg/ml DNA and 4 μl/ml of Lipofectamine reagent. After 5–7 h of exposure to the transfection medium, cells were refed with construct-free serum-containing medium and incubated 12–24 h before experiments. The transfection efficiency was consistently >30% with the Lipofectamine reagents.

Western blot analysis. Cells were scraped from 10-cm petri dishes and collected into 15-ml tubes, centrifuged, and washed two times with cold PBS. Cell pellets were resuspended in 20–100 μl of lysis buffer [1% Triton X-100, 150 mM NaCl, 5 mM EDTA, and 50 mM Tris·HCl (pH 7.4)] supplemented with 1× protease inhibitor cocktail (Sigma) and 100 μg/ml PMSF before use. Cells were incubated in the lysis buffer for 30 min on ice. The cell lysates were then centrifuged at 14,000 rpm for 15 min, and the insoluble fraction was discarded. The protein concentrations in the supernatant were determined by the Coomassie Plus protein assay (Pierce) with BSA as a standard. Proteins (20 μg) were mixed and boiled in SDS-PAGE sample buffer for 2 min. The protein samples separated on 8% SDS-PAGE were then transferred to nitrocellulose membranes by electroblotting in a Mini Trans-Blot cell transfer apparatus (Bio-Rad) according to the manufacturer's instructions. After incubation for 1 h at 22–24°C in a blocking buffer (0.1% Tween 20 in PBS) containing 5% nonfat dry milk powder, the membranes were incubated with a polyclonal rabbit anti-Kv1.5 antibody (Alomone Labs) overnight at 4°C. The membranes were then washed with the blocking buffer and incubated with corresponding horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. After unbound antibodies were washed with the blocking buffer, the bound antibodies were detected with an enhanced chemiluminescence detection system (Amersham).

Electrophystological measurement. Whole cell K^+ currents were recorded with an Axopatch-1D amplifier and a DigiData 1200 interface (Axon Instruments) with patch-clamp techniques (43). Patch pipettes (2–3 MΩ) were fabricated on an electrode puller (Sutter) with borosilicate glass tubes and fire polished on a microforge (Narishige). Command voltage protocols and data acquisition were performed with pCLAMP 8 software (Axon Instruments). All experiments were performed at room temperature (22–24°C). For recording optimal whole cell $I_{K(V)}$, a coverslip containing cells was positioned in a recording chamber and superfused (2–3 ml/min) with the standard extracellular (bath) solution, which contained (in mM) 141 NaCl, 4.7 KCl, 1.8 $CaCl_2$, 1.2 $MgCl_2$, 10 HEPES, and 10 glucose (pH 7.4). For the Ca^{2+}-free solution, $CaCl_2$ was replaced by equimolar $MgCl_2$ and 1 mM EGTA was added to chelate residual Ca^{2+}. The pipette (internal) solution for recording whole cell $I_{K(V)}$ contained (in mM) 135 KCl, 4 $MgCl_2$, 10 HEPES, 10 EGTA, and 5 Na_2ATP (pH 7.2). The green fluorescence emitted at 507 nm was used to visualize the cells transfected with *KCNA5*-GFP or pCMS-EGFP constructs.

Nuclear morphology determination. Cells grown on 25-mm coverslips were washed with PBS, fixed in 95% ethanol for 15 min at −20°C, and stained with 100 μM 4',6-diamidino-2-phenylindole dihydrochloride (DAPI, Sigma) for 8 min at 24°C. The blue fluorescence emitted at 461 nm was used to visualize the cell nuclei. The DAPI-stained cells were examined with a Nikon fluorescence microscope, and the cell (nuclear) images were acquired with a high-resolution Solamere fluorescence imaging system. For each coverslip, 15–25 fields (with 20–40 cells in each of the fields) were randomly selected to determine the percentage of apoptotic cells in total cells based on the morphological characteristics of apoptosis. Cells with clearly defined nuclear breakage, remarkably condensed nuclear fluorescence, and significantly shrunken cell nuclei were defined as apoptotic cells.

Measurement of caspase-3 activity. The protein samples were prepared and protein concentration was measured as for Western blot analysis. Proteins (40 μg) were diluted by the lysis buffer to a final volume of 50 μl and subjected to caspase-3 measurements with a caspase-3 colorimetric assay kit (Assay Designs, Ann Arbor, MI), following the instructions provided by the manufacturer. Briefly, 75 μl of a caspase-3 substrate was added to 50 μl of the protein sample in a microtiter plate. The caspase-3 substrate, Ac-DEVD, was labeled with the chromophore *p*-nitroaniline (pNA). A colorimetric substrate (Ac-DEVD-pNA) releases free pNA from the substrate on cleavage by DEVDase. Free pNA produces a yellow color that was monitored by a spectrophotometer at 405 nm after 3 h of incubation of the plate at 37°C. The amount of yellow color produced on cleavage is proportional to the amount of caspase-3 activity present in the sample.

Cell volume evaluation. The cell volume (V) is proportional to the area (S) and radius (r) of the inscribed circle of a cell as estimated by the following equation: $V \propto S \times r$. Consequently, cell geometry allows us to evaluate cell volume changes by measuring the cell surface area (which is similar to the area of the inscribed circle because the cultured cells attached onto coverslips are very flat) on the cell images acquired with a high-resolution Solamere fluorescence imaging system. Only transfected cells, visualized by green fluorescence, were used for measurement of the cell surface area with Kodak 1D 3.6 software. Furthermore, a decrease in the inscribed circle area in a cell not only reflects cell volume decrease but also indicates a progression of cell "rounding" (less adherence), which is another characteristic of AVD and apoptosis.

To determine and compare the changes of cell volume in control and *KCNA5*-transfected cells, the cell surface area values measured after treatment with staurosporine (ST) were normalized to the area value before ST treatment and expressed as a percentage of the initial area value. Using percent changes of cell volume to compare AVD in

control and *KCNA5*-transfected cells also minimizes the potential errors stemming from variation of cell sizes.

Chemicals. ST (Sigma) was prepared as a 1 mM stock solution in DMSO; aliquots of the stock solution were then diluted 1,000–2,000 times to the culture media for experiments. 4-Aminopyridine (4-AP; Sigma) was directly dissolved in the culture media or bath solutions on the day of use. The membrane-permeant DAPI was prepared as a 10 mM stock solution in an antibody buffer containing 500 mM NaCl, 20 μM NaN_3, 10 μM $MgCl_2$, and 20 μM Tris·HCl (pH 7.4) and diluted 1:100 in PBS before use.

Statistics. The composite data are expressed as means ± SE. Statistical analysis was performed with paired or unpaired Student's *t*-test or ANOVA and post hoc tests (Student-Newman-Keuls) where appropriate. Differences were considered to be significant at $P < 0.05$.

RESULTS

Functional expression of human KCNA5 gene. To define an optimal time for electrophysiological and fluorescent microscopy experiments, we first determined the time course of KCNA5 protein expression in COS-7 cells and rat PASMC transiently transfected with *KCNA5*. As shown in Fig. 1*A*, KCNA5 protein was heterologously expressed at a very high level in both cell types transfected with *KCNA5* construct compared with cells transfected with an empty vector. The expression level of KCNA5 protein was maximal 24 h after transfection and was maintained for up to 48 h (Fig. 1*A*, *a* and *b*). The heterologous KCNA5 protein level in KCNA5-transfected COS-7 cells and rat PASMC (24 h after transfection) was 34 and 14 times greater, respectively, than the endogenous KCNA5 protein levels in cells transfected with a control or empty vector (a pCMS-EGFP vector without *KCNA5*).

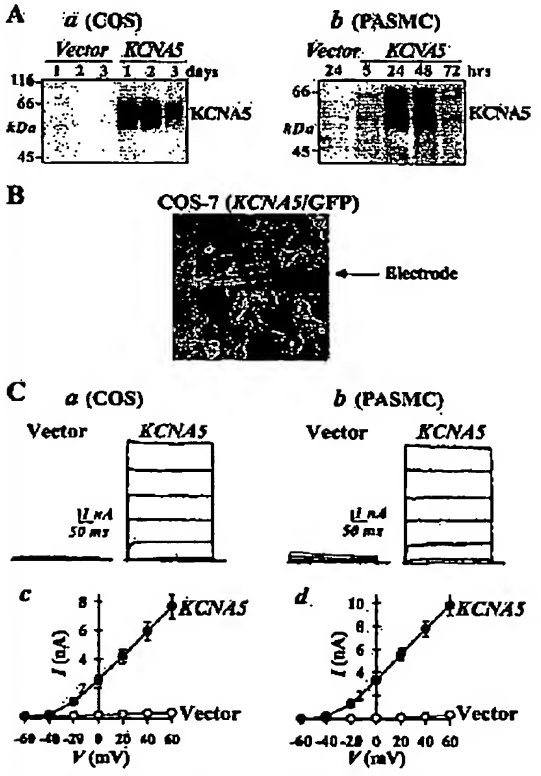

Fig. 1. Expression and functional characterization of the human KCNA5 channels in COS-7 cells and rat pulmonary artery smooth muscle cells (PASMC). *A*: Western blot analysis of KCNA5 protein levels in COS-7 cells (*a*) and rat PASMC (*b*) 1–3 days after transfection with the control pCMS-enhanced green fluorescent protein (EGFP) vector (vector) and *KCNA5*. *B*: representative image of cells showing a *KCNA5*-GFP-transfected COS-7 cell that was patched for recording K^+ currents. *C*: representative currents (*a* and *b*), elicited by depolarizing the cells from a holding potential of −80 mV to test potentials ranging from −60 to +60 mV, and composite current-voltage (*I-V*) relationships (*c* and *d*) in control (vector) and *KCNA5*-transfected cells.

To characterize the function of heterologous KCNA5, whole cell $I_{K(V)}$ were recorded and compared in cells transiently transfected with the control vector and the *KCNA5*-GFP construct. Twenty-four to thirty-two hours after transfection, the cells emitting green fluorescence were selected for recording $I_{K(V)}$ (Fig. 1*B*). As shown in Fig. 1*C*, the whole cell $I_{K(V)}$, elicited by depolarizing the cells from a holding potential of −70 mV to a series of test potentials ranging from −60 to +60 mV, were significantly increased in *KCNA5*-transfected COS-7 cells (Fig. 1*Ca*) and rat PASMC (Fig. 1*Cb*); the amplitude of $I_{K(V)}$ was increased by 22–29 times compared with the empty vector-transfected COS cells (Fig. 1*Cc*) and rat PASMC (Fig. 1*Cd*). These results show that *1*) the whole cell $I_{K(V)}$ in the *KCNA5*-transfected cells are dominantly generated by the heterologous KCNA5 channels, whereas the contribution of endogenous K^+ channels to the total $I_{K(V)}$ is minimal, and *2*) the optimal time for maximal expression of KCNA5 channels is 24–48 h.

Overexpression of KCNA5 accelerates AVD. Increased KCNA5 channel expression and subsequent augmentation of $I_{K(V)}$ would promote loss of intracellular K^+ and enhance AVD induced by apoptosis inducers such as ST. To investigate whether overexpression of *KCNA5* influences ST-induced cell volume decrease, we first transfected COS-7 cells and rat PASMC with the control vector (pCMS-EGFP construct) and the *KCNA5*-GFP construct. Twenty-four hours after transfection the cells were treated with 1 μM ST for 30–150 min, and the cells emitting green fluorescence (representing transfected cells with either control vector or *KCNA5*-GFP construct) were selected for cell volume measurement (Fig. 2*A*).

Treatment with 1 μM ST for 30–120 min caused significant cell shrinkage in COS-7 cells (Fig. 2*Ba*) and rat PASMC (Fig. 2*Bb*); the maximal decrease in cell volume was 46.3% and 61.5%, respectively. The ST-induced AVD was significantly accelerated in *KCNA5*-transfected COS-7 cells (Fig. 2*Ba*) and rat PASMC (Fig. 2*Bb*) compared with the control GFP vector-transfected cells. For example, the time to reach EC_{50} for ST-induced AVD was shortened from 47.2 ± 4.1 ($n = 26$) to 22.1 ± 2.2 ($n = 26$) min ($P < 0.001$) in COS-7 cells and from 46.5 ± 4.5 ($n = 19$) to 24.2 ± 3.6 ($n = 17$) min in rat PASMC by overexpression of *KCNA5* (Fig. 2*B*). These results suggest that increased whole cell $I_{K(V)}$ in *KCNA5*-transfected cells promote apoptotic cell shrinkage.

Overexpression of KCNA5 gene enhances apoptosis. To examine whether *KCNA5* overexpressed in mammalian cells influences apoptosis, we first transfected COS-7 cells with the control vector and the *KCNA5* expression construct. Twenty-four hours after transfection, the cells were treated with vehicle (DMSO) or ST. The percentage of cells that exhibited apoptotic nuclear morphology (i.e., nuclear condensation, shrink-

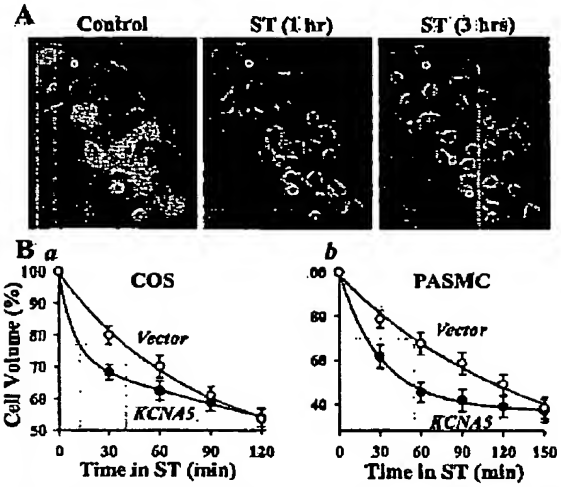

Fig. 2. Apoptotic volume decrease (AVD) is accelerated in *KCNA5*-transfected cells. *A*: rat PASMC transfected with the control pCMS-EGFP vector before (control) and after treatment with 1 μM staurosporine (ST) for 1 and 3 h. *Center* and *right*, phase contrast images of the cells. *Left*, combination of the phase contrast image overlapping with a fluorescent image of GFP. The photographs were taken at a magnification of ×20. The cells emitting green fluorescence were selected for the cell volume measurements. *B*: summarized data showing the decrease in cell volume in COS-7 cells (*a*) and rat PASMC (*b*) transfected with the control vector and *KCNA5* before (0 min) and during treatment with 1 μM ST for 30–150 min. The time-course curves for ST-mediated cell volume decrease in control and *KCNA5*-transfected cells are significantly different ($P < 0.01$) in COS-7 cells and rat PASMC.

age, and breakage) was then determined by fluorescence microscopy (Fig. 3*Aa*).

In COS-7 cells, overexpression of *KCNA5* alone had little effect on basal apoptosis, the percentage of cells undergoing apoptosis in cells that were not treated with ST (10.6 ± 0.9% in control cells vs. 12.6 ± 1.5% in *KCNA5*-transfected cells; $P = 0.26$; Fig. 3*Ab*), whereas ST-induced apoptosis in *KCNA5*-transfected cells (21.3 ± 2.5%; $n = 24$) was almost two times greater than in control cells (11.9 ± 1.9%; $n = 25$, $P < 0.01$) (Fig. 3*A*, *b* and *c*). The basal apoptosis was determined 24 h after transfection, when the transfection level of *KCNA5* was maximal. In rat PASMC, however, overexpression of *KCNA5* alone significantly increased the basal apoptotic rate (determined 24 h after transfection) from 8.2 ± 1.1% (control cells) to 21.3 ± 2.0% ($P < 0.001$) (Fig. 3*Ba*), in addition to enhancing ST-induced apoptosis (from 31.3 ± 1.5% to 52.3 ± 3.2%; $P < 0.05$; Fig. 3*Bb*). These results suggest that overexpression of KCNA5 channels increases whole cell $I_{K(V)}$, accelerates ST-induced AVD, and enhances ST-induced apoptosis in COS-7 cells and rat PASMC, whereas *KCNA5* overexpression alone makes rat PASMC prone to undergo apoptosis.

Caspase-3 activation is increased in KCNA5-transfected cells. Cleavage of procaspase-3 to generate the active effector caspase-3 is an important step that leads to chromatin degradation and ultimately to apoptosis (13). To confirm that the apoptotic morphological changes that we observed in the previous experiments are associated with caspase-3 activation, we measured and compared the caspase-3 activity in total protein samples obtained from the control vector- and *KCNA5*-transfected COS-7 cells and rat PASMC (24 h after transfection). Consistent with the effect on apoptosis (Fig. 3), overexpression of *KCNA5* had a negligible effect on basal caspase-3 activity in COS-7 cells (194 ± 30 vs. 212 ± 20 U/mg total protein in vector- and *KCNA5*-transfected cells) but significantly increased basal caspase-3 activity in rat PASMC (152 ± 19 vs. 234 ± 19 U/mg; $P < 0.05$) (Fig. 4).

Treatment of the cells with 1 μM ST for 6 h markedly increased caspase-3 activity in both COS-7 cells and rat PASMC, and the ST-mediated caspase activation was significantly greater in *KCNA5*-transfected COS-7 cells (622 U/mg) than control vector-transfected cells (416 U/mg; $P < 0.05$) (Fig. 4). The reason we could not detect a significant difference

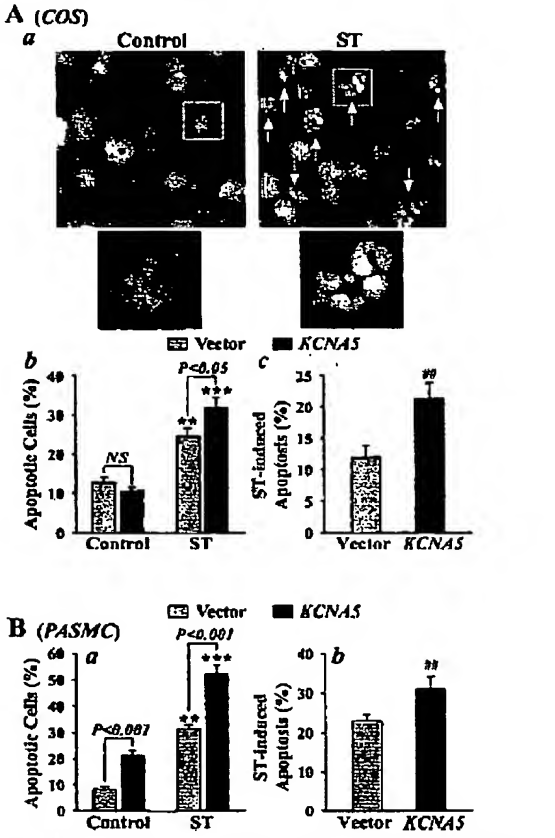

Fig. 3. Apoptosis is enhanced in *KCNA5*-transfected cells. *A*: 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI)-stained nuclei (*a*) of COS-7 cells treated with (ST) or without (control) 1 μM ST for 3 h. Arrows indicate the apoptotic nuclei (photographs were taken at a magnification of ×40). Magnified images (*bottom*) show a normal nucleus (*left*) and an apoptotic nucleus (*right*). *b*: Summarized data showing % of apoptotic cells before (control) and after (ST) 3-h treatment with 1 μM ST in COS-7 cells transfected with the control pCMS-EGFP vector and *KCNA5*. $^{**}P < 0.01$, $^{***}P < 0.001$ vs. control. NS, no statistical significance ($P > 0.5$). *c*: Normalized increase of % of apoptotic cells after treatment with ST showing that ST-induced apoptosis is significantly greater in *KCNA5*-transfected cells ($n = 25$) than in control cells (vector; $n = 24$). $^{\#\#}P < 0.01$ vs. vector. *B*: summarized data (*a*) showing % of apoptotic cells before (control) and after (ST) 1.5-h treatment with 0.5 μM ST in rat PASMC transfected with the control vector and *KCNA5*. $^{**}P < 0.01$, $^{***}P < 0.001$ vs. control. *b*: Normalized increase of % of apoptotic cells after treatment with ST in control ($n = 10$) and *KCNA5*-transfected ($n = 10$) rat PASMC. $^{\#\#}P < 0.01$ vs. vector.

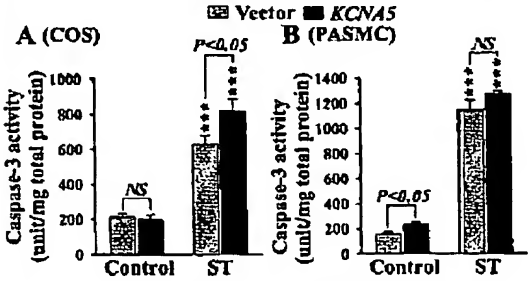

Fig. 4. Increased caspase-3 activation in *KCNA5*-transfected cells. Summarized data show the caspase-3 activity in total cell protein samples obtained from COS-7 cells (*A*) and rat PASMC (*B*) transfected with the control pCMS-EGFP vector and *KCNA5* before (control) and after (ST) 6-h treatment with 1 μM ST. ***$P < 0.05$ vs. control.

in ST-induced caspase-3 activation between control vector- and *KCNA5*-transfected rat PASMC was probably a high basal level of caspase-3 activity in *KCNA5*-transfected cells.

Blockade of KCNA5 channels decelerates ST-induced AVD and inhibits ST-induced apoptosis. Overexpression of *KCNA5* increased whole cell $I_{K(V)}$, accelerated AVD, enhanced caspase-3 activation, and induced apoptosis. To verify that the proapoptotic effect of *KCNA5* overexpression is due to increased K^+ efflux or cytoplasmic K^+ loss, we examined the effect of 4-AP, a Kv channel blocker, on KCNA5 currents and ST-induced AVD and apoptosis. Extracellular application of 3 mM 4-AP significantly and reversibly decreased whole cell $I_{K(V)}$ in *KCNA5*-GFP-transfected COS-7 cells (Fig. 5, *A* and *C*) and rat PASMC (Fig. 5, *B* and *D*), indicating that 4-AP is a potent blocker of KCNA5 channels. In these experiments, whole cell KCNA5 currents were recorded in *KCNA5*-GFP-transfected cells both superfused and dialyzed with Ca^{2+}-free solutions.

We then investigated whether 4-AP-mediated blockade of KCNA5 channels influences ST-induced AVD and apoptosis. Empty vector-transfected and *KCNA5*-GFP-transfected (Fig. 6*A*) rat PASMC (24 h after transfection) were first treated with 3 mM 4-AP for 30 min and then treated with 1 μM ST for 30–150 min in the presence of 4-AP. As shown in Fig. 6, inhibition of KCNA5 channel activity with 4-AP markedly attenuated or decelerated ST-induced AVD in both vector-transfected (Fig. 6*Ba*) and *KCNA5*-transfected (Fig. 6*Bb*) rat PASMC. The differences of ST-induced AVD (calculated by subtracting the time course curves in cells without 4-AP treatment from the curves in cells with 4-AP treatment) in vector- or *KCNA5*-transfected cells indicate that 4-AP-mediated inhibition of AVD is much greater in *KCNA5*-transfected cells than in vector-transfected cells at 30–90 min of ST treatment (Fig. 6*C*). In control vector-transfected cells, for example, 4-AP attenuated AVD from 63.1 ± 1.0% ($n = 38$) to 76.2 ± 1.1% ($n = 50$; a 21% inhibition) 60 min after ST treatment, whereas in *KCNA5*-GFP-transfected cells, 4-AP decreased AVD from 46.2 ± 1.3% ($n = 33$) to 67.2 ± 1.2% ($n = 36$; a 45% inhibition) (Fig. 6*D*). These results further demonstrate that 4-AP-sensitive native Kv channels in rat PASMC contribute to ST-induced AVD and overexpressed KCNA5 channels are responsible for the acceleration or augmentation of ST-induced AVD in *KCNA5*-transfected rat PASMC.

To examine whether blockade of KCNA5 channels with 4-AP affects ST-induced apoptosis, the *KCNA5*-GFP-transfected COS-7 cells (24 h after transfection) were first treated with 3 mM 4-AP for 30 min and then treated with 1 μM ST for 3 h in the presence of 4-AP. Inhibition of KCNA5 channel activity with 4-AP markedly inhibited ST-induced apoptosis (Fig. 7*A*). In control *KCNA5*-GFP-transfected cells, treatment with 1 μM ST increased the percentage of apoptotic cells from 12.1 ± 0.8% to 41.2 ± 4.0% (a 3.4-fold increase), whereas in *KCNA5*-GFP-transfected cells treated with 4-AP, ST increased the percentage of apoptotic cells from 13.7 ± 0.9% to 29.4 ± 2.5% (a 2.1-fold increase) (Fig. 7*A*). The ST-induced apoptosis in *KCNA5*-GFP-transfected cells was reduced by ~46% after treatment of the cells with 4-AP (from 29.1 ± 4.0% to 15.7 ± 2.5%; $n = 7$; $P < 0.01$; Fig. 7*B*). These results suggest that the increase in $I_{K(V)}$ due to overexpressed KCNA5 channels results in the enhancement of ST-induced apoptosis.

It is noted that treatment with 4-AP, a potent blocker of native Kv channels and KCNA5 channels, only blocked 46% of ST-induced apoptosis (Fig. 7*B*). The remaining 54% of apoptosis induced by ST may result from *1*) activation of 4-AP-sensitive K^+ channels that were not completely blocked by the dose of 4-AP we used in these experiments, *2*) activation of 4-AP-insensitive K^+ channels and other cation or anion (e.g., Cl^-) channels, and *3*) a possible apoptotic effect of 4-AP per se.

DISCUSSION

Pulmonary vascular medial hypertrophy, an important pathological feature in patients with pulmonary hypertension,

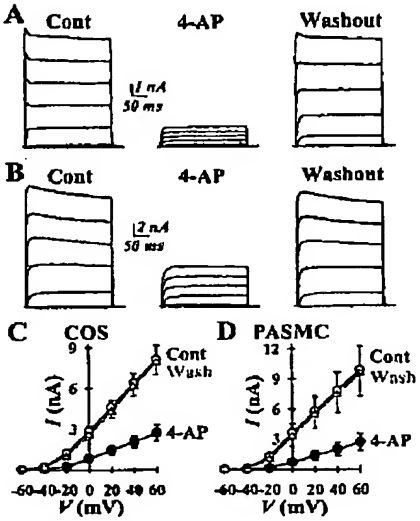

Fig. 5. Inhibitory effect of 4-aminopyridine (4-AP) on whole cell K^+ currents in *KCNA5*-transfected cells. *A* and *B*: representative currents, elicited by depolarizing the cells from a holding potential of −80 mV to a series of test potentials ranging from −60 to +60 mV in 20-mV increments, in *KCNA5*-transfected COS-7 cells (*A*) and rat PASMC (*B*) before (Cont), during (4-AP), and after (washout) extracellular application of 3 mM 4-AP. *C* and *D*: composite *I-V* relationships (means ± SE) from *KCNA5*-transfected COS-7 cells (*C*; $n = 9$) and rat PASMC (*D*; $n = 7$) before, during, and after 4-AP treatment.

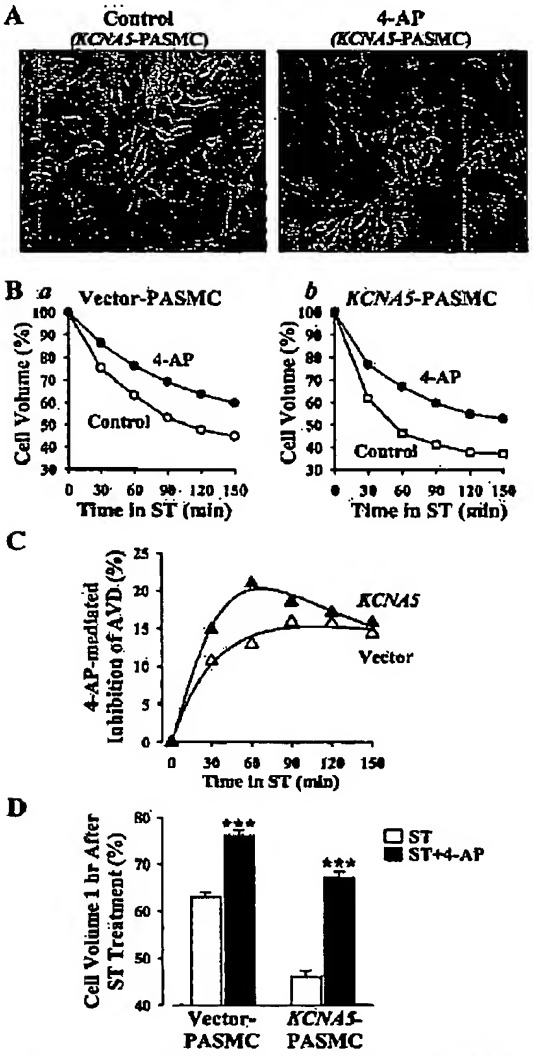

Fig. 6. Functional blockade of KCNA5 channels by 4-AP decelerates ST-induced AVD. *A*: representative images showing rat PASMC (24 h after transfection with *KCNA5*-GFP) with (4-AP) or without (control) pretreatment with 3 mM 4-AP. These cells were not treated with ST. Only transfected cells, recognized by green fluorescence, were used to measure volume changes before and after treatment with ST. *B*: summarized data (means ± SE) showing the % decrease in cell volume of vector (*a*)- and *KCNA5* (*b*)-transfected PASMC in response to 1 μM ST (treated for 30–150 min) in the absence (control) and presence (4-AP) of 3 mM 4-AP. *C*: differences of 4-AP-mediated inhibition of AVD (subtracting the time-course curve in control cells from the curve in 4-AP-treated cells) in vector- and *KCNA5*-transfected cells. *D*: % changes (mean ± SE) of cell volume in PASMC treated with and without 4-AP 1 h after ST treatment for vector- and *KCNA5*-transfected cells. $^{***}P < 0.001$ vs. ST alone. The 4-AP-mediated inhibition of ST-induced AVD is significantly greater in KCNA5-transfected cells than in vector-transfected cells.

is mainly due to unbalanced PASMC proliferation and apoptosis. Increased PASMC growth and/or decreased PASMC apoptosis can concurrently mediate thickening of the pulmonary vascular wall, subsequently reducing the lumen diameter of pulmonary arteries, increasing pulmonary vascular resistance, and raising pulmonary arterial pressure (1, 9, 10, 28, 29, 35, 48). Precise control of the balance of cell apoptosis and proliferation in PASMC thus plays a critical role in maintaining *1*) the normal structural and functional integrity of the pulmonary vasculature and *2*) the low pulmonary arterial pressure in normal subjects. In animal experiments, it has been demonstrated that inducing apoptosis of hypertrophied PASMC in intact pulmonary vessels can prevent the progression of the medial hypertrophy (9, 10, 28). Therefore, it is important to define the genes and gene products that participate in regulating PASMC apoptosis and proliferation.

Apoptotic cell shrinkage, an incipient prerequisite for apoptosis that precedes most other morphological alterations and caspase activation during the apoptotic process, results from a loss of cytosolic ions (e.g., K^+ and Cl^-) and water in response to apoptosis inducers (21, 24). Therefore, the transmembrane K^+ transport and activity of Kv channels play an important role in the regulation of AVD (4, 5, 11, 18–21, 23, 24, 36, 38, 40, 41). In addition to regulating cell volume, K^+ in the cytosol also serves as an inhibitor of caspases and nucleases (14), the central executioners of the apoptotic pathway (13). In other words, maintaining a high $[K^+]_c$ (i.e., ~140 mM) is necessary for both the maintenance of normal cell volume or K^+ homeostasis and the suppression of caspases and nucleases (5, 11, 14). Activation of K^+ channels in the plasma membrane increases K^+ efflux or loss and plays an important role in initiating AVD and apoptosis, whereas blockade of K^+ channels inhibits the apoptotic cell shrinkage and attenuates apoptosis induced by a variety of apoptosis inducers, such as ST, valinomycin, anti-Fas, tumor necrosis factor-α, H_2O_2, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP), and ultraviolet radiation (4, 5, 11, 18–21, 23, 24, 36, 38–41). These results suggest that cytosolic K^+ homeostasis and sarcolemmal K^+ channel activity are both involved in the regulation of apoptosis.

The inability of 4-AP, a potent blocker of Kv channels in vascular smooth muscle cells, to abolish ST-induced apoptosis suggests that AVD or apoptosis is not only regulated by 4-AP-sensitive Kv (e.g., KCNA5) channels but also regulated by 4-AP-insensitive K^+ channels as well as Cl^- channels. In other words, multiple mechanisms are involved in regulating apoptotic cell shrinkage and apoptosis; activity of Kv channels may serve as one of the important mechanisms to regulate programmed cell death.

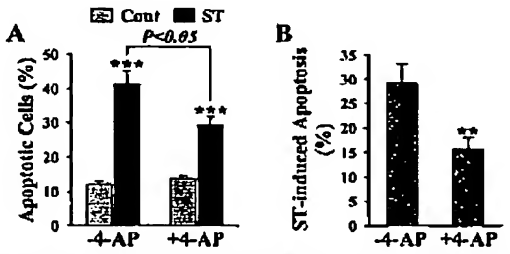

Fig. 7. Functional blockade of KCNA5 channels by 4-AP inhibits ST-induced apoptosis. *A*: summarized data showing % of apoptotic cells before (Cont) and after (ST) 3-h treatment with 1 μM ST in *KCNA5*-transfected COS-7 cells in the absence (−4-AP; $n = 7$) or presence (+4-AP; $n = 7$) of 3 mM 4-AP. $^{***}P < 0.001$ vs. Cont. *B*: normalized increase of % of apoptotic cells after treatment with ST showing that ST-induced apoptosis is significantly inhibited by 4-AP in *KCNA5*-transfected cells. $^{**}P < 0.01$ vs. −4-AP.

Downregulated and dysfunctional Kv channels have been implicated in PASMC from patients with IPAH (42, 46). Acute hypoxia decreases Kv channel activity and chronic hypoxia downregulates Kv channel expression in PASMC, suggesting that hypoxia mediates pulmonary vasoconstriction and vascular medial hypertrophy by, in part, inhibiting Kv channel activity (8, 15, 26, 33, 38, 44). The decreased Kv currents due to downregulated expression and/or attenuated Kv channel function depolarize PASMC, open voltage-dependent Ca^{2+} channels, promote Ca^{2+} influx, increase $[Ca^{2+}]_{cyt}$, and ultimately cause pulmonary vasoconstriction and stimulate PASMC proliferation (22, 25, 32, 43). The inhibited Kv channels in PASMC (42, 46) may also be involved in the attenuated PASMC apoptosis in IPAH patients (48) and subsequently contribute to the excessive pulmonary arterial medial hypertrophy observed in these patients.

A common hypothesis is that enhanced PASMC proliferation and inhibited PASMC apoptosis both contribute to pulmonary vascular medial hypertrophy. Therefore, inhibition of PASMC proliferation and induction of apoptosis in hypertrophied PASMC may both be beneficial for treatment of severe pulmonary arterial hypertension (9, 10, 28). For example, NO and prostacyclin (PGI_2) are potent endothelium-derived vasodilators and inhibitors of smooth muscle cell growth (7, 16, 17, 30). Short-term infusion of PGI_2 and inhalation of NO decrease pulmonary vascular resistance, whereas long-term therapy with PGI_2 improves survival in IPAH patients (1). Furthermore, NO induces apoptosis in vascular smooth muscle cells (6, 19, 30, 34, 37). In PASMC, both NO and PGI_2 activate K^+ channels (e.g., voltage-gated, Ca^{2+}-activated, and ATP-sensitive K^+ channels) (2, 45). These results suggest that activation of sarcolemmal K^+ channels may serve as an important therapeutic target for pulmonary hypertension because of its *1*) vasodilative effect on pulmonary arteries by causing membrane hyperpolarization, closing voltage-dependent Ca^{2+} channels, attenuating Ca^{2+} influx, and decreasing $[Ca^{2+}]_{cyt}$ in PASMC, *2*) antiproliferative effect on PASMC by reducing cytoplasmic and nuclear $[Ca^{2+}]$, and *3*) proapoptotic effect on PASMC by inducing apoptotic volume decrease and facilitating caspase activation. Inhibition of proliferation or induction of apoptosis in "misguided" hypertrophied PASMC leads to the regression of pulmonary medial hypertrophy (9, 10, 28).

As mentioned above, downregulation of Kv channel α-subunit (e.g., KCNA5) expression and inhibition of Kv channel function in PASMC have been implicated in IPAH and hypoxia-mediated pulmonary arterial hypertension (1–3, 27, 42, 46). In animal experiments, Pozeg et al. (27) showed that in vivo gene transfer of KCNA5, an important pore-forming α-subunit that forms delayed-rectifier Kv channels (8, 15), increased $I_{K(V)}$ in PASMC, decreased pulmonary vascular thickness, reduced pulmonary vascular resistance, and lowered pulmonary arterial pressure. These results provide compelling evidence that overexpression of Kv channels in PASMC is an efficient approach for treatment of pulmonary arterial hypertension.

In summary, we showed in this study that in vitro overexpression of human *KCNA5* in COS-7 cells and rat PASMC increases whole cell $I_{K(V)}$, accelerates ST-induced apoptotic cell shrinkage, and enhances ST-induced caspase-3 activation and apoptosis. Functional blockade of KCNA5 channels with 4-AP reduced $I_{K(V)}$ and inhibited ST-induced apoptosis in COS-7 cells, confirming that the proapoptotic effect of *KCNA5* overexpression is due to an increased K^+ efflux. Furthermore, overexpression of the human *KCNA5* in rat PASMC induced "basal" apoptosis or, in other words, made PASMC inclined to undergo apoptosis in the absence of apoptosis inducers. These results suggest that, compared with COS-7 cells, PASMC may rely more on K^+ channel activity and the apoptotic process to remove unnecessary (e.g., misguided or hypertrophied) cells under normal conditions to maintain a thin vascular wall. Genetic abnormalities (e.g., bone morphogenetic protein receptor II mutations) and *KCNA5* downregulation and dysfunction may lead to the removal or inhibition of the K^+ channel-dependent apoptotic process, thereby contributing to the development of pulmonary vascular medial hypertrophy.

Further studies are necessary to determine whether the apoptotic effect of *KCNA5* overexpression occurs in normal human PASMC and whether overexpression of Kv channels in PASMC from IPAH patients is able to restore normal K^+ function and facilitate apoptosis. The results from this study also suggest that normal expression and function of KCNA5 channels are not only necessary for maintaining and regulating resting membrane potential and $[Ca^{2+}]_{cyt}$ (1–3, 8, 15, 43, 47) but also essential for promoting cells to undergo apoptosis. The therapeutic effect of *KCNA5* gene transfer on pulmonary arterial hypertension (27) may be partially due to enhanced PASMC apoptosis, which leads to the regression of pulmonary vascular remodeling and reduction of pulmonary vascular resistance.

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GRANTS

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REFERENCES

1. **Archer S and Rich S.** Primary pulmonary hypertension: a vascular biology and translational research "work in progress". *Circulation* 102: 2781–2791, 2000.
2. **Archer SL, Huang JM, Hampl V, Nelson DP, Shultz PJ, and Weir EK.** Nitric oxide and cGMP cause vasorelaxation by activation of a charybdotoxin-sensitive K channel by cGMP-dependent protein kinase. *Proc Natl Acad Sci USA* 91: 7583–7587, 1994.
3. **Archer SL, Souil E, Dinh-Xuan AT, Schremmer B, Mercier JC, El Yaagoubi A, Nguyen-Huu L, Reeve HL, and Hampl V.** Molecular identification of the role of voltage-gated K^+ channels, Kv1.5 and Kv2.1, in hypoxic pulmonary vasoconstriction and control of resting membrane potential in rat pulmonary artery myocytes. *J Clin Invest* 101: 2319–2330, 1998.
4. **Bock J, Szabo I, Jekle A, and Gulbins E.** Actinomycin D-induced apoptosis involves the potassium channel Kv1.3. *Biochem Biophys Res Commun* 295: 526–531, 2002.
5. **Bortner CD, Hughes FM Jr, and Cidlowski JA.** A primary role for K^+ and Na^+ efflux in the activation of apoptosis. *J Biol Chem* 272: 32436–32442, 1997.
6. **Chiche JD, Schlutsmeyer SM, Bloch DB, de la Monte SM, Roberts JD Jr, Filippov G, Janssens SP, Rosenzweig A, and Bloch KD.** Adenovirus-mediated gene transfer of cGMP-dependent protein kinase increases the sensitivity of cultured vascular smooth muscle cells to the antiproliferative and pro-apoptotic effects of nitric oxide/cGMP. *J Biol Chem* 273: 34263–34271, 1998.
7. **Clapp LH, Finney P, Turcato S, Tran S, Rubin LJ, and Tinker A.** Differential effects of stable prostacyclin analogs on smooth muscle

proliferation and cyclic AMP-generation in human pulmonary artery. *Am J Respir Cell Mol Biol* 26: 194–201, 2002.
8. **Coppock EA, Martens JR, and Tamkun MM.** Molecular basis of hypoxia-induced pulmonary vasoconstriction: role of voltage-gated K^+ channels. *Am J Physiol Lung Cell Mol Physiol* 281: L1–L12, 2001.
9. **Cowan KN, Heilbut A, Humpl T, Lam C, Ito S, and Rabinovitch M.** Complete reversal of fatal pulmonary hypertension in rats by a serine elastase inhibitor. *Nat Med* 6: 698–702, 2000.
10. **Cowan KN, Jones PL, and Rabinovitch M.** Regression of hypertrophied rat pulmonary arteries in organ culture is associated with suppression of proteolytic activity, inhibition of tenascin-C, and smooth muscle cell apoptosis. *Circ Res* 84: 1223–1233, 1999.
11. **Dallaporta B, Hirsch T, Susin SA, Zamzami N, Larochette N, Brenner C, Marzo I, and Kroemer G.** Potassium leakage during the apoptotic degradation phase. *J Immunol* 160: 5605–5615, 1998.
12. **Hounstetter A and Izumo S.** Apoptosis: basic mechanisms and implications for cardiovascular disease. *Circ Res* 82: 1111–1129, 1998.
13. **Hengartner MO.** The biochemistry of apoptosis. *Nature* 407: 770–776, 2000.
14. **Hughes FMJ, Bortner CD, Purdy GD, and Cidlowski JA.** Intracellular K^+ suppresses the activation of apoptosis in lymphocytes. *J Biol Chem* 272: 30567–30576, 1997.
15. **Hulme JT, Coppock EA, Felipe A, Martens JR, and Tamkun MM.** Oxygen sensitivity of cloned voltage-gated K^+ channels expressed in the pulmonary vasculature. *Circ Res* 85: 489–497, 1999.
16. **Ishida A, Sasaguri T, Kosaka C, Nojima H, and Ogta J.** Induction of the cyclin-dependent kinase inhibitor p21(Sdi1/Cip1/Waf1) by nitric oxide-generating vasodilator in vascular smooth muscle cells. *J Biol Chem* 272: 10050–10057, 1997.
17. **Kolpakov V, Gordon D, and Kulik TJ.** Nitric oxide-generating compounds inhibit total protein and collagen synthesis in cultured vascular smooth muscle cells. *Circ Res* 76: 305–309, 1995.
18. **Krick S, Platoshyn O, Sweeney M, Kim H, and Yuan JX-J.** Activation of K^+ channels induces apoptosis in vascular smooth muscle cells. *Am J Physiol Cell Physiol* 280: C970–C979, 2001.
19. **Krick S, Platoshyn O, Sweeney M, McDaniel SS, Zhang S, Rubin LJ, and Yuan JX-J.** Nitric oxide induces apoptosis by activating K^+ channels in pulmonary vascular smooth muscle cells. *Am J Physiol Heart Circ Physiol* 282: H184–H193, 2002.
20. **Lang F, Busch GL, Ritter M, Volkl H, Waldegger S, Gulbings E, and Haussinger D.** Functional significance of cell volume regulatory mechanisms. *Physiol Rev* 78: 247–306, 1996.
21. **Maeno E, Ishizaki Y, Kanaseki T, Hazama A, and Okada Y.** Normotonic cell shrinkage because of disordered volume regulation is an early prerequisite to apoptosis. *Proc Natl Acad Sci USA* 97: 9487–9492, 2000.
22. **Means AR.** Calcium, calmodulin and cell cycle regulation. *FEBS Lett* 347: 1–4, 1994.
23. **Nietsch HH, Roe MW, Fiekers JF, Moore AL, and Lidofsky SD.** Activation of potassium and chloride channels by tumor necrosis factor α. Role in liver cell death. *J Biol Chem* 275: 20556–20561, 2000.
24. **Okada Y, Maeno E, Shimizu T, Dezaki K, Wang J, and Morishima S.** Receptor-mediated control of regulatory volume decrease (RVD) and apoptotic volume decrease (AVD). *J Physiol* 532: 3–16, 2001.
25. **Platoshyn O, Golovina VA, Bailey CL, Limsuwan A, Krick S, Juhaszova M, Seiden JE, Rubin LJ, and Yuan JX-J.** Sustained membrane depolarization and pulmonary artery smooth muscle cell proliferation. *Am J Physiol Cell Physiol* 279: C1540–C1549, 2000.
26. **Post JM, Hume JR, Archer SL, and Weir EK.** Direct role for potassium channel inhibition in hypoxic pulmonary vasoconstriction. *Am J Physiol Cell Physiol* 262: C882–C890, 1992.
27. **Pozeg ZI, Michelakis ED, McMurtry MS, Thebaud B, Wu XC, Dyck JR, Hashimoto K, Wang S, Moudgil R, Harry G, Sultanian R, Koshal A, and Archer SL.** In vivo gene transfer of the O_2-sensitive potassium channel Kv1.5 reduces pulmonary hypertension and restores hypoxic pulmonary vasoconstriction in chronically hypoxic rats. *Circulation* 107: 2037–2044, 2003.
28. **Rabinovitch M.** Elastase and the pathobiology of unexplained pulmonary hypertension. *Chest* 114: 213S–224S, 1998.
29. **Riley DJ, Thakker-Varia S, Wilson FJ, Poiani GJ, and Tozzi CA.** Role of proteolysis and apoptosis in regression of pulmonary vascular remodeling. *Physiol Res* 49: 577–585, 2000.
30. **Roberts JD Jr, Chiche JD, Weimann J, Steudel W, Zapol WM, and Bloch KD.** Nitric oxide inhalation decreases pulmonary artery remodeling in the injured lungs of rat pups. *Circ Res* 87: 140–145, 2000.
31. **Rono JR and Loyd JE.** Primary pulmonary hypertension. *Lancet* 361: 1533–1544, 2003.
32. **Santella L.** The role of calcium in the cell cycle: facts and hypotheses. *Biochem Biophys Res Commun* 244: 317–324, 1998.
33. **Smirnov SV, Robertson TP, Ward JPT, and Aaronson PI.** Chronic hypoxia is associated with reduced delayed rectifier K^+ current in rat pulmonary artery muscle cells. *Am J Physiol Heart Circ Physiol* 266: H365–H370, 1994.
34. **Smith JD, McLean SD, and Nakayama DK.** Nitric oxide causes apoptosis in pulmonary vascular smooth muscle cells. *J Surg Res* 79: 121–127, 1998.
35. **Stenmark KR and Mecham RP.** Cellular and molecular mechanisms of pulmonary vascular remodeling. *Annu Rev Physiol* 59: 89–144, 1997.
36. **Trimarchi JR, Liu L, Smith PJS, and Keefe DL.** Apoptosis recruits two-pore domain potassium channels used for homeostatic volume regulation. *Am J Physiol Cell Physiol* 282: C588–C594, 2002.
37. **Wang BY, Ho HK, Lin PS, Schwarzacher SP, Pollman MJ, Gibbons GH, Tsao PS, and Cooke JP.** Regression of atherosclerosis: role of nitric oxide and apoptosis. *Circulation* 99: 1236–1241, 1999.
38. **Wang J, Juhaszova M, Rubin LJ, and Yuan X-J.** Hypoxia inhibits gene expression of voltage-gated K^+ channel α subunits in pulmonary artery smooth muscle cells. *J Clin Invest* 100: 2347–2353, 1997.
39. **Wang L, Xu D, Dai W, and Lu L.** An ultraviolet-activated K^+ channel mediates apoptosis of myeloblastic leukemia cells. *J Biol Chem* 274: 3678–3685, 1999.
40. **Wang X, Xiao AY, Ichinose T, and Yu SP.** Effects of tetraethylammonium analogs on apoptosis and membrane currents in cultured cortical neurons. *J Pharmacol Exp Ther* 295: 524–530, 2000.
41. **Yu SP, Yeh CH, Sensi SL, Gwag BJ, Canzoniero LMT, Farhangrazi ZS, Ying HS, Tian M, Dugan LL, and Choi DW.** Mediation of neuronal apoptosis by enhancement of outward potassium current. *Science* 278: 114–117, 1997.
42. **Yuan JX-J, Aldinger AM, Juhaszova M, Wang J, Conte JVJ, Gaine SP, Orens JB, and Rubin LJ.** Dysfunctional voltage-gated K^+ channels in pulmonary artery smooth muscle cells of patients with primary pulmonary hypertension. *Circulation* 98: 1400–1406, 1998.
43. **Yuan X-J.** Voltage-gated K^+ currents regulate resting membrane potential and $[Ca^{2+}]_i$ in pulmonary arterial myocytes. *Circ Res* 77: 370–378, 1995.
44. **Yuan X-J, Goldman WF, Tod ML, Rubin LJ, and Blaustein MP.** Hypoxia reduces potassium currents in cultured rat pulmonary but not mesenteric arterial myocytes. *Am J Physiol Lung Cell Mol Physiol* 264: L116–L123, 1993.
45. **Yuan X-J, Tod ML, Rubin LJ, and Blaustein MP.** NO hyperpolarizes pulmonary artery smooth muscle cells and decreases the intracellular Ca^{2+} concentration by activating voltage-gated K^+ channels. *Proc Natl Acad Sci USA* 93: 10489–10494, 1996.
46. **Yuan X-J, Wang J, Juhaszova M, Gaine SP, and Rubin LJ.** Attenuated K^+ channel gene transcription in primary pulmonary hypertension. *Lancet* 351: 726–727, 1998.
47. **Yuan X-J, Wang J, Juhaszova M, Golovina VA, and Rubin LJ.** Molecular basis and function of voltage-gated K^+ channels in pulmonary arterial smooth muscle cells. *Am J Physiol Lung Cell Mol Physiol* 274: L621–L635, 1998.
48. **Zhang S, Fantozzi I, Tigno DD, Yi ES, Platoshyn O, Thistlethwaite PA, Kriett JM, Yung G, Rubin LJ, and Yuan JX-J.** Bone morphogenetic proteins induce apoptosis in human pulmonary vascular smooth muscle cells. *Am J Physiol Lung Cell Mol Physiol* 285: L740–L754, 2003.
49. **Zimmermann KC, Bonzon C, and Green DR.** The machinery of programmed cell death. *Pharmacol Ther* 92: 57–70, 2001.

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application	:	Walter Schubert
Application No.	:	10/664,678
Filed	:	September 12, 2003
For	:	USES OF SUBSTANCES WITH IMMUNOMODULATING ACTIVITY FOR THE TREATMENT OF AMYOTROPHIC LATERAL SCLEROSIS
Examiner	:	CROWDER, Chun
Attorney's Docket	:	S&H-010DX

Group Art Unit: 1644

* *

I hereby certify that this correspondence is being sent via facsimile transmission to: Examiner Chun Crowder, Group Art Unit 1644, Fax No. (571) 273-8300 on 2-8-6.

By: [signature]
Charles L. Gagnebin III
Registration No. 25,467
Attorney for Applicant(s)

* *

DECLARATION OF WALTER SCHUBERT, M.D.
UNDER 37 C.F.R. §1.132

I, Walter Schubert, M.D., a citizen of Germany, residing at Am Mühlengrund 9, D-39175 Biederitz, Germany, declare the following:

1. I received my doctoral degree in Experimental Biology at the University of Bonn, Germany. I have been an associate professor at the Medical Neurobiology Department at the Otto-von-Guaricke-University of Magdeburg in Germany since 1994. I am also the founder and CEO of the company MELTEC, which was founded in 1999.

2. I specialized in neurology and psychiatry from 1979 to 1985 while I was attending the University Clinic of Neurology in Bonn, Germany, and was elected the principal investigator of a laboratory for

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neuromuscular disorder in 1983 to 1988. I then joined the Centre of Molecular Biology (ZMBH) at the University of Heidelberg, Germany, as a research associate. I have extensive experience in clinical neurology, neuroimmunology, neuropathology and molecular cell biology.

3. I am also an inventor of the subject matter set forth in the present, above-identified patent application.

4. I have read and am familiar with the prosecution history of the present application, including the USPTO Office Action dated August 8, 2005.

5. This declaration is a supplement to the Response submitted separately herewith, providing information relevant to whether it would be predictable that amyotrophic lateral sclerosis (ALS) could be treated by administering soluble Fcγ-RI, Fcγ-RII, and/or Fcγ-RIII receptors to effect Fc receptor blockade, that the specification as filed enables a person skilled in the art to make and use the invention, that the operativeness of the claimed invention is reasonably predictable based on *in vitro* studies cited in the specification, that operativeness remains reasonably predictable based on most recent medical research references, and to establish what the inventor, as an ordinary skilled artisan, knew at the time of the filing of the application.

6. The Office Action rejects claims 1-7 under 35 U.S.C. §112, first paragraph, as being non-enabling for the subject matter described in the specification. In particular, the Examiner states that, while the specification is enabling for a method of blocking the cytotoxic activity of FcγRIII-positive, ALS-specific cells in a patient with ALS using soluble FcγRIII, the specification "does not reasonably provide enablement for the full breath (sic) of soluble Fcγ receptors and a method of treating a patient with ALS" (Par. 7)."

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7. I respectfully disagree with the Examiner's assessment that the specification is non-enabling for a method of treating ALS by administering an effective amount of soluble Fcγ receptors, wherein said soluble Fcγ receptors bind to immunoglobulin in said patient to treat amytrophic lateral sclerosis. First, the method is enabled in the specification for all classes (i.e., RI, RII and RIII) of soluble Fcγ receptors as much as for the one class of soluble FcγRIII receptors. The entire group of Fcγ receptors (all three classes) is identified in the disclosure, as well as the source and derivation of each class sufficient for a skilled molecular biologist to obtain any of the group, and appropriate doses are given for an initial therapeutic scheme in Example 1, from which a skilled practitioner could derive a therapeutic scheme for each and every soluble Fcγ receptor in the group. Methods of producing the requisite soluble Fcg receptors were known in the art prior to the filing date sufficiently for a trained molecular biologist to obtain the substances (e.g., see Sautes et al., 1994). The Example 1 in the disclosure teaches FcgRs generally, produced from *E. Coli* fusion proteins. With regard to therapeutic regimen, Example 1 does not merely show a toleration test, as stated by the Examiner; rather, the Example directs a therapeutic scheme whereby one begins with a toleration test, and then administers "subsequently 150 mg/weight kg daily, over a period of 5 days." A therapeutic endpoint is specifically called out in the listing of solution advantages #3: "... the therapeutic success can not only be determined clinically, but also on the cellular level, ..., by the examination of blood samples for therapy-related decrease in number, or even a total elimination, of the number of the Fcγ receptor-positive cellular forms in the blood stream." (see the Specification at page 7, numbered paragraph 3). This therapeutic scheme and goal provides sufficient guidance and direction for a skilled medical doctor to treat ALS patients with the method of the claimed invention.

8. Attached herewith as APPENDIX A, is a report generated for the purpose of responding to the Examiner's rejections in prior Office Actions. This report explains in detail that ALS-specific immune cells are characterized by specific protein combinations, which combinations identify Fcγ receptor as key proteins. Moreover, the report also provides results of comparison between occurrence of Fcγ receptor in the immune cells of normal individuals versus occurrence in immune cells of patients with ALS.

9. The research upon which my company was founded pertains to toponomic fingerprinting, which is a whole-cell protein fingerprinting (WCPF) technology that uses an automated 3D visualization system (MELK) integrated with Meltec's biomathematical tools. The MELK system detects protein networks, identifies and localizes at the cellular level, thereby deciphering cellular function. Using this technology, spatial organization of proteins can be determined, which is important for the functional code of a cell. Cellular functionalities are encoded by a finite and highly non-random repertoire of toponome units (TUs) which is a system of rules to construct topological hierarchies in a cell's proteome. The entirety of these TUs represents the total functional code of a cell.

10. Toponomic Fingerprinting (TF) is based upon the theory that a cell is made up almost entirely by protein-complexes in all compartments and in the cytosol. Using tag libraries, toponome enciphers the complete functional plan of a cell or a tissue. It is composed of highly non-random spatial assemblies of proteins, the functional toponome unit (FTUs). There are clear-cut rules for the local formation of FTUs in cells, which, when deciphered by the TF at a large scale, unravel the hierarchies of proteins and directly lead to the detection of key target proteins. This functional organization of proteins is inaccessible when the cellular structures are destroyed, as for example, by large scale expression profiling procedures. TF is highly specific

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for a cell type, a functional state of a cell, e.g., as in disease, or a tissue. TF is a characteristic collection of combinatorial protein patterns (CPP) which contain protein clusters.

11. To acquire data, an automated imaging cycler MELK (Multi-Epitope-Ligand-"Kartograph") is used for "massive parallel toponome screening," which allows for the automated running of Repetitive-Incubation-Imaging-Bleaching-Cycles (RIIBC). As described in Appendix A, starting on page 6, after several dye-conjugated antibody binding, fluorescence images are registered and stored creating an illustration of protein networks. As described on Appendix A, starting on page 7, using our own proprietary software, image processing allows for image correction, threshold setting, signal alignments and object segmentation creating an information of mapping cellular or subcellular toponomic fingerprints.

12. In combination with statistical analysis, a particular algorithm called the "motif-finder" that we developed is used to automatically identify CPP motifs with high statistical significance among different comparison groups, e.g., control v. treatment groups. A leading protein is identified as the key player (potential target protein). As explained in Appendix A, starting on page 7, all CPPs within the TF are expressed as a combinatorial binary code in which each protein is denoted as absent or present above a threshold level (0/1). A fixed "reading frame" (x-axis) contains all theoretically possible CPPs. The CPP as a binary code can be transformed into a decimal number on the x-axis. This decimal number is plotted against the frequency of the CPP's (y-axis) in the sample. The resulting TF contain all occurring CPPs as well as "silent areas," which indicate that the corresponding theoretical CPPs on the reading frame do not occur in the biological system measured. The combination of occurring CPPs and silent areas together make up the TF of a cell system. These TF pinpoint differences between treated and untreatd biological samples, or

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between different cell systems or diseases. From this, CPP clusters are identified which one protein common to all CPPs within the cluster is particularly identified.

13. Methodology and results of identifying cell clusters from the venous blood of 7 healthy individuals and from 9 sporadic ALS patients are shown starting on page 11 of Appendix A. We used a library of 18 different monoclonal antibodies directly conjugated to a dye (FITC) as well as the MELK technology as outlined in the method section of Appendix A. The antibodies were all directed against well-characterized cell surface proteins, most of them belonging to cell surface adhesion receptors and cell surface proteolytic enzymes. Table 4, on page 17 of Appendix A, provides a total list of ALS-specific motifs, i.e., most-significant abnormal, cell-surface, protein clusters in ALS based on an analysis from our proprietary pattern recognition-algorithm including our motif-finder. Table 5, on page 20 of Appendix A, provides a comparison of the most striking findings for normal control individuals and ALS patients. Upon a 2D and 3D analysis, the leading protein found to be significantly frequent was CD16 (FCγRIII) in ALS patients. Each protein has a unique distribution pattern over the cell surface mononuclear cells upon 3D visualization. While the data indicate that CD16 is the leading protein in both healthy and ALS blood cells, the motifs were found inherently different. In the control group, CD16 co-clusters with CD11b/CD2 or hladr; if CD16 co-clusters with hladr, the following molecules are always absent - CD11b, CD621, CD36 and CD4. This indicates that the inverse correlation of cell surface receptors is important for the normal state of mononuclear cells. In contrast, in ALS, CD16 co-clusters with hladr and also with CD36 or CD36 and CD621, but never with CD11b. In addition, CD16 in ALS does also co-cluster with CD45ra. These clusters strictly exclude hladr and CD11b. Thus, ALS-specific cell surface protein clusters indicate presence of abnormal cell surface differentiation. Moreover, based on the high statistical

significance, CD16 can be indicated as a valid peripheral biomarker for ALS and a basis for CD16-oriented modification therapy.

14. Table 1 in the Specification of the present application presents some of the results of the research discussed above. It is logical and reasonable to predict with confidence, from these results disclosed in the specification, that a soluble Fcg receptor introduced to the serum in order to selectively bind the constant gamma chain of immunoglobulin in the serum would thereby selectively deprive the FcgRIII receptors on the ALS-disease-specific immune cells from binding with said immunoglobulin, and thus would block activation of these disease-specific immune cells.

15. Soluble FcγRI, FcγRII and FcγRIII receptors operate essentially the same with respect to the invention insofar as they are all capable of binding the gamma-chain region on immunoglobulin in the blood. Therefore, the three classes each operate in similar fashion to prevent blood-borne IgG from subsequently activating the FcγRIII receptor-positive, ALS-disease-specific immune cells.

16. Recent research supports the claimed method of treating autoimmune aspects of ALS by introducing soluble FcγRs. The role of activating FcγRs in providing a critical link between ligands and effector cells in type II and type III inflammation is now well established (Ravetch and Bolland, 2001). Nakamura et al., 2005, state that the activating-type Fc receptors (FcRs) are essential for the development of autoimmune diseases, suggesting that regulation of inhibitory or activating FcR is an ideal target as a therapeutic agent(p.169), that macrophages lacking FcγRI or FcRγ show impaired phagocytosis, owing to co-engagement of FcγRs during phagocytosis effectively triggering activation of the Src tyrosine-kinase family, which phosphorylates ITAM (p. 175), and that recent studies using FcR-

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deficient mice also reveal that the development of autoimmune diseases depends upon FcγRs (p.178, citing Heyman 2000; Ravetch and Solland, 2001; Takai 2002; and Hogarth, 2002). Suates-Fridman et al. (2003) state that the Fc receptors for IgG (FcγR) play a critical role in immunity by linking the IgG antibody mediated responses with cellular effector and regulatory functions of the immune system (p.148), that data obtained in mice have led to the concept that many systemic autoimmune diseases are under FcγR control (p. 150), and that not only are FcγR important molecules that mediate and control the effector functions of IgG antibodies, but they also control the autoimmunity-tolerance balance in the periphery (p. 151).

17. The McGeer article cited by the USPTO Examiner for the conclusion that there is no effective ALS treatment exists reviews a set of therapeutic schemes that neither includes nor is related to the therapeutic strategy of the invention. Between the years 1965 and 2005, immunosuppressant strategies in clinical trials have only tested administration of cyclosporine, prednisolone, azathiprene, cylophosphadine and radiation [See Clark et al., 2005, pp. 128-304). In a recent study that examined whether FcγR-mediated processes can contribute to these effects of ALS patient immunoglobulins, it was found that FcγRs appear to participate in IgG uptake into motor neurons as well as IgG-mediated increases in intracellular calcium and acetylcholine release from motor axon terminals (Mohamed, 2002). In 2006, soluble Fcγ receptors are still categorized as "new leads" by the ALS Therapy Development Foundation (see attachment, ALS TDF Web page 2006).

I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001

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of Title 18 of the Unites States Code, and that such willful false statements so made may jeopardize the validity of the document, or application, or any patent issuing thereon.

Signed this 7th day of February, 2006.

By: _____
Walter Schubert, M.D.

ATTACHED:

APPENDIX A: MELTEC REPORT
APPENDIX B: REFERENCES

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APPENDIX B: REFERENCES

Sautes et al., 1994.
Recombinant soluble Fc gamma receptors: production, purification and biological activities. Sautes C, Galinha A, Bouchard C, Mazieres N, Spagnoli R, Fridman WH. J Chromatogr B Biomed Appl. 1994 Dec 9;662(2):197-207.
http://

Nakamura et al., 2005.
Fc receptor targeting in the treatment of allergy, autoimmune diseases and cancer. Nakamura, A., K. Akiyama, and T. Takai. Expert Opin. Ther. Targets (2005) 9(1):169-190
http://

Clark et al., 2005:
"Amyotrophic Lateral Sclerosis: A report on the state of research into the cause, cure, and prevention of ALS," June 2005; J. Clark, C. Pritchard, S. Sunak; Prepared for the Dept. of Public Health, State of Massachusetts by the ALS Therapy Development Foundation;
http://www.als.net/docs/ALSReport.pdf

ALS Web page: (Copy attached)
http://

Ravetch and Bolland, 2001
IGG FC RECEPTORS. Jeffrey V. Ravetch and Silvia Bolland. *Annual Review of Immunology*. Vol. 19: 275-290 (Volume publication date April 2001); (doi:10.1146/annurev.immunol.19.1.275)
http://

[CITED IN NAKAMURA et al.:]
Heyman B., Regulation of antibody responses via antibodies, complement, and Fc receptors. *Ann. Rev. Immunol.* (2000) 18:709-737
http://

Takai, T., Roles of Fc receptors in autoimmunity. *Nat. Rev. Immunol.* (2000) 2(8):580-592
http://

Hogarth, P.M., Fc receptors are major mediators of antibody based inflammation in autoimmunity. *Curr. Opin. Immunol.* (2002) 14(6):798-802.
http://

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Sautes-Fridman et al., 2003
Fc Gamma Receptors: A magic link with the outside world. Catherine Sautes-Fridman, Lydie Cassard, Joel Cohen-Solal and Wolf-Herman Fridman. *ASHI Quarterly, 4th Quarter*, 2003, p. 148-151
http://_____

Mohamed et al., 2002
Immunoglobulin Fc gamma receptor promotes immunoglobulin uptake, immunoglobulin-mediated calcium increase, and neurotransmitter release in motor neurons. Mohamed HA, Mosier DR, Zou LL, Siklos L, Alexianu ME, Engelhardt JI, Beers DR, Le WD, Appel SH. *J Neurosci Res.* 2002 Jul 1;69(1):110-6.
http://_____

ALSO SEE:

http://www.biology-online.org/dictionary/fc_receptors

http://www.lef.org/protocols/prtcl-008.shtml#autoimmunedisease

http://www.med.nus.edu.sg/phys/Projects_FcgR_Alirio.htm

http://www.als.net/research/treatments/treatmentDetail.asp?treatmentID=993

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